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Review

Gene editing nuclease and its application in tilapia

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ABSTRACT

Gene editing nucleases including zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) system (CRISPR/Cas9) provide powerful tools that improve our ability to understand the physiological processes and their underlying mechanisms. To date, these approaches have already been widely used to generate knockout and knockin models in a large number of species. Fishes comprise nearly half of extant vertebrate species and provide excellent models for studying many aspects of biology. In this review, we present an overview of recent advances in the use of gene editing nucleases for studies of fish species. We focus particularly on the use of TALENs and CRISPR/Cas9 genome editing for studying sex determination in tilapia.

1. Introduction

Reverse genetics approaches are critical for exploring gene function and genetic engineering. Traditionally, generating a gene editing model was largely dependent on the availability of embryonic stem cells (ESCs) that has been developed in only a few model organisms, such as mouse and rat [1,2]. Homologous recombination (HR) based gene editing in mice opened the door to genome editing. Due to the lack of methods for HR and especially the development of ESCs, there has been no effective technology for generating gene editing in non-rodent organisms. Now, this situation is thoroughly changed with the recent development of engineered nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs) and clustered, regularly interspaced, short palindromic repeats (CRISPR)–CRISP R-associated (Cas) systems (CRISPR/Cas9), which have been used to develop genome editing models precisely in a wide variety of organisms from invertebrates to vertebrates [3–5]. Teleosts, which make up roughly 50% of the extant vertebrates, provide important models for studying many aspects of biology [6]. For instance, zebrafish and medaka have clearly shown their utility for studies of fundamental processes underlying development and disease. Cichlids, including tilapia, are valuable organisms for studying speciation, behavior and the evolution of sex determination [7]. Most importantly, with the advances in high-throughput sequencing approaches, whole genomes and transcriptomes of a large number of fishes have been published [7–9]. The subsequent step is to dissect the information encoded in these genome sequences. Therefore, it is necessary to establish efficient and precise gene editing approach that enables investigators to determine how genotype influences phenotype. In recent years, successful applications of ZFN, TALEN and CRISPR/Cas9 in several fish species have been reported [5,10], which greatly improve both basic and applied research in fish biology. In this review, we summarize the use of gene editing nucleases in fishes focusing mainly on the application of TALEN and CRISPR/Cas9 in tilapia (*Oreochromis niloticus*) genome engineering, sex determination and sex control for aquaculture.

2. Gene editing nucleases

ZFNs are composed of a customized DNA-binding C_2H_2 zinc fingers and the DNA cleavage domain of the restriction enzyme Fok I. A zinc finger was designed and assembled to recognize a nucleotide triplet, and multiple zinc fingers are joined in tandem to bind the target sequence. Two ZFNs are required to generate double strand break (DSB) in a desired target locus because the Fok I cleavage domain must be dimerized (Fig. 1a) [4,10]. The development of ZFN technology enhances our ability to introduce mutations or insertions in any animals [11,12]. However, some limitations still constrain the ability to create high quality of zinc finger domains that recognize any desired sequences [3]. Similar to ZFN, TALEN, a new genome editing technology based on the transcription activator-like effectors (TALEs) from *Xanthomonas* plant pathogens,

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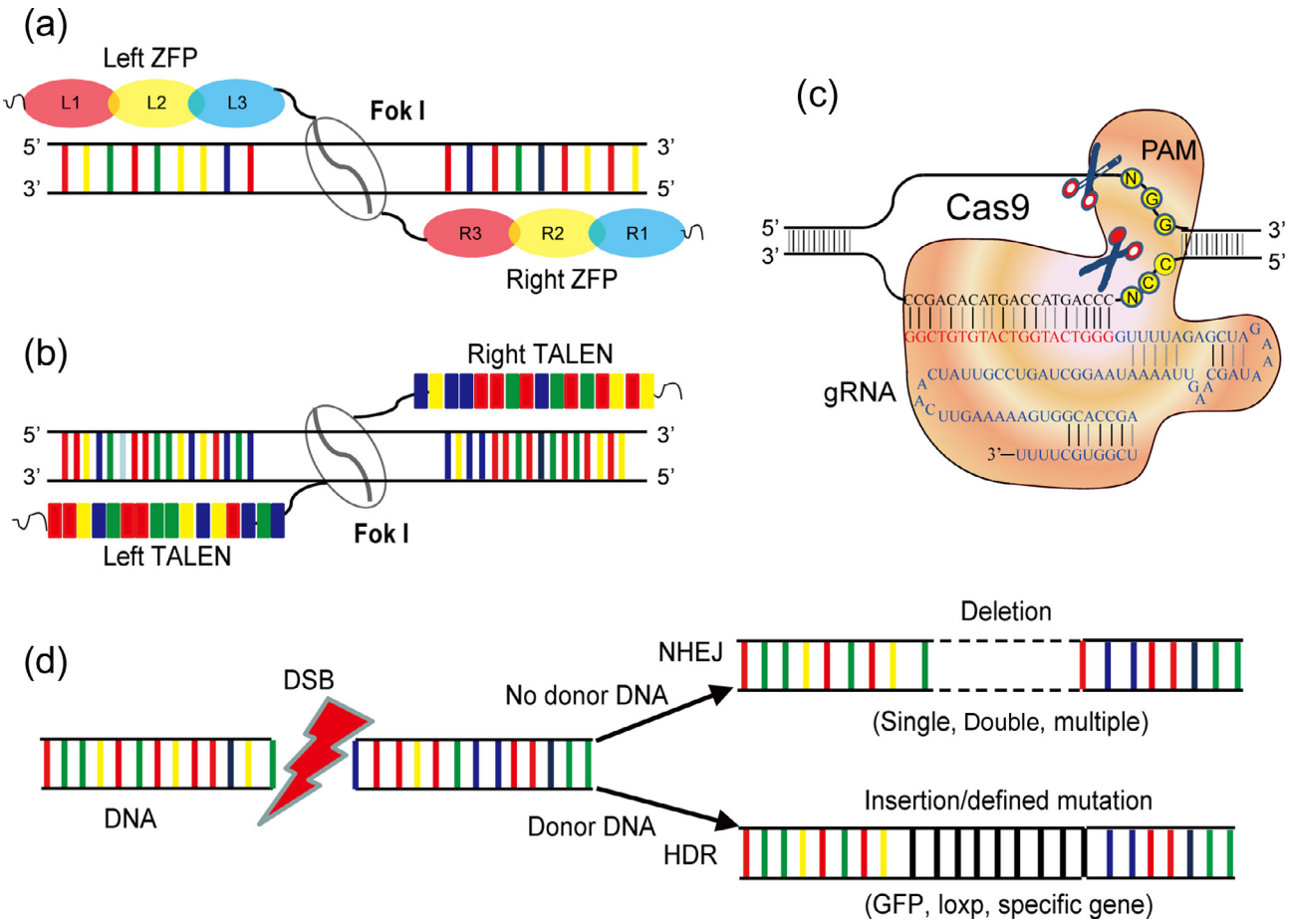

Fig. 1. (Color online) Schematics summarizing the mechanisms of gene editing nucleases. **a** ZFN mediated genome editing. ZFN target sites consist of two zinc-finger binding sites separated by a 5–7 bp spacer sequence recognized by the Fok I cleavage domain. ZFNs recognize DNA using 3 bp recognition motifs (ZFPs). Several ZFPs in tandem can give specificity to a specific sequence. Two ZFNs recognize adjacent sequences, each of which is fused to half of the obligate dimer Fok I nuclease. **b** TALEN mediated genome editing. TALENs bind to DNA through a highly conserved 34 amino acid TAL effector repeat domain. Two TALENs are used to cut DNA. **c** CRISPR/Cas9 mediated genome editing. Cas9 binds to specific DNA sequences via the base-pairing of the gRNA. The target is present immediately upstream of a Protospacer Adjacent Motif (PAM). **d** Nuclease induced DSB can be repaired by NHEJ or HDR pathway. Imprecise NHEJ-mediated repair can produce insertion and deletion mutations at the site of the DSB. HDR-mediated repair can introduce precise insertions from a single- or double-stranded DNA donor template. ZFP, zinc finger protein; gRNA, guide RNA; DSB, double strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair.

is also comprised of a programmable DNA binding domain fused to the Fok I and function as dimers to generate DSB [13,14]. Each TALE repeat can bind to a single base pair with high specificity associated with its 12th and 13th repeat-variable di-residues. Theoretically, TALE repeats can be joined in tandem to bind any DNA sequences (Fig. 1b) [15,16]. Therefore, TALEN is superior to ZFN because of its simplified design and assembly strategies, such that creation of effective TALEN protein is significantly faster and cheaper than ZFN [4,17].

Recently, an even simpler and more efficient RNA-guided gene editing technology, the type II CRISPR/Cas9, has been developed to rapid and efficient target DNA sequence. It is based on the *Streptococcus pyogenes* adaptive immune system which requires three components, the nuclease Cas9, a targeting crRNA and a *trans*-activating crRNA (tracrRNA) [18,19]. Further improvement of this system was performed by engineering dual crRNA: tracrRNA to form a single guide RNA (gRNA), which contain a designed hairpin that mimics the crRNA–tracrRNA complex, as it is sufficient to direct Cas9 protein to cleave DNA sequence (Fig. 1c). Different from ZFN and TALEN using DNA-binding proteins to recognize DNA sequence, CRISPR/Cas9 utilizes 20 bp long gRNA to bind its target via Watson–Crick base pairing. Additionally, the protospacer-adjacent motif (PAM, NGG) immediately downstream of the target sequence together with gRNA determine the specificity of this system [18–20]. Importantly, CRISPR/Cas9 is capable of editing multiple genes simultaneously, producing conditional alleles, and generating tagged protein with high efficiency [19]. Compared with ZFN and TALEN, CRISPR/Cas9 is found to be much easier, less expensive, much simpler, more efficient technology to precisely modify the genome of any organisms during development [5,21].

DSB induced by these nucleases is commonly repaired by two pathways, non-homologous end joining (NHEJ) or homology-directed repair (HDR) [21]. NHEJ pathway is an error-prone process that can create disruptive insertions or deletions through which the encoding protein loses its function if the disruptions occur in the exons. The HDR pathway uses additional homologous DNA fragments as templates for repair, which can be exploited to precisely modify a desired locus or insert exogenous DNA, such as GFP and LoxP (Fig. 1d) [22].

3. Applications of gene editing nucleases in fish species

Previously, mutants have been generated by ethyl nitrosourea (ENU) and targeting induced local lesions in genomes (TILLING) methods in medaka, fugu and zebrafish [23–25]. Nonetheless, the subsequent identification of the molecular lesion is laborious and

involves time-consuming genetic mapping. In addition, it is extremely difficult to obtain homozygous mutants in some aquaculture fishes due to the long period of sexual maturity. Therefore, it is necessary to identify genes of interest and obtain mutants followed by the observation of the phenotypic effects after loss of their function in F_0 generation. In recent years, ZFN, TALEN and CRISPR/Cas9 are emerging as promising new methods to perform genome editing and have been successfully exploited in several fish species including zebrafish [10,13,20], medaka [26–28], tilapia [29,30], rainbow trout [31], yellow catfish [32,33], Atlantic salmon [34], southern catfish [35], common carp [36], channel catfish [37], killifish [38,39], blind Mexican cavefish [40], and rohu [41] (Table 1). The high frequencies of mutations induced by these gene editing nucleases can produce obvious phenotypes in F_0 founders [29,30,35,36]. For instance, in common carp, disruption of *sp7a* and *sp7b* individually resulted in severe bone defects and the *mstnba* mutant fish have grown significantly more muscle cells in the F_0 generation [36]. In southern catfish, germ cell meiosis was preciously initiated in the testis after disruption of *cyp26a1* [35]. In blind Mexican cavefish, loss of *oca2* resulted in mosaic loss of melanin pigmentation visible as albino patches, suggesting biallelic gene mutations in F_0 founder fish [41]. Of course, the mutations generated by engineering nucleases can also be transmitted to next generation to produce homozygous mutants. Germline transmission has been reported in zebrafish, medaka, tilapia, yellow catfish and rainbow trout (Table 1). A number of stable heritable homozygous mutants have been established in these fishes. For example, homozygous mutation of *gsdf* in XY medaka leads to sex reversal [42]. In addition, many genes are functionally redundant, and thus the influences of loss of a single gene can be compensated by another one. It is worth noting that CRISPR/Cas9 has greatly facilitated the targeted inactivation of several genes simultaneously in a number of organisms including some fishes, overcoming the issues of redundancy [36,43]. This is especially noteworthy for teleost fish since it has undergone an additional genome duplication event during its evolution, such as *cyp19a1a* and *cyp19a1b*. Furthermore, conditional gene knockout was also performed in zebrafish which could be used to generate models for studying gene function in temporal manners or specific tissue [44,45], which is also valuable for other aquacultures. Overall, these versatile platforms now permit us to elucidate fundamental questions of interest precisely in fishes.

Transgenic fish are important for scientific research and production of new fish lines. The first successful gene transfer in fishes was the transfer of the growth hormone gene to produce transgenic goldfish [46]. Subsequently, transgenic fishes were successfully produced in rainbow trout [47], channel catfish [48], salmon [49], common carp [50] and tilapia [51]. The technologies commonly used in fish, including Tol2 and Sleeping Beauty (SB) transposon systems, typically result in random insertion of DNA into the genome at multiple sites [52–54]. Accumulated information showed that the random integration of exogenous DNA fragments still has limitations in fish genetic engineering. The genomic environment surrounding the integration site can influence the expression of the transgene [55,56]. Although site-specific recombinase systems, such as FLP/FRT, Cre/LoxP and Dre/rox, have been used to generate transgenic models in mouse and zebrafish [57], these technologies have not been widely applied to fish species. Recently, gene editing nucleases have significantly improved our ability to insert exogenous DNA fragments into specific genomic loci. Gene editing nuclease mediated HDR has been demonstrated efficiently to insert transgenes into specific genome loci in many organisms without using any site specific recombinases or ESCs, several fish species including zebrafish, medaka and killifish [20,27,39]. For example, targeted knockin of GFP or LoxP fragments in a defined locus was achieved in zebrafish [45,58]. Therefore, site-specific gene integration provides a powerful tool for producing site specific transgene in fish to improve important aquaculture traits, avoiding many of the adverse effects of conventional technologies [59]. Taken together, the development of loss-of-function and gain-of-function technologies based on these engineered nucleases increases our ability to understand the molecular mechanisms underlying specific characteristics [60].

4. Genetically modified tilapia by gene editing nucleases

4.1. Tilapia genome editing

The Nile tilapia, a gonochoristic teleost with a stable XX/XY male heterogametic sex determination system, is one of the most important farmed fish species [61]. Numerous studies have been conducted to investigate the molecular mechanisms of its sex determination [61]. This was greatly motivated by the higher growth rates of all male progenies because males grow about 50% faster than females. Tilapia is also an attractive laboratory model for elucidating the molecular mechanism of sex determination with several advantages. First, all XX fish can be obtained by crossing a wild type XX female with a pseudomale (XX male) that producing functional sperm after sex reversal by hormonal treatment. All XY fish were produced by crossing a wild type female (XX) with a supermale (YY) [62]. Second, the whole genome of Nile tilapia has been published [7]. Finally, several gonadal transcriptomes at four critical developmental stages have been sequenced, which provide a better understanding of the early stage in the process of sex determination and differentiation [63]. Previously, the

Table 1
Schematic overview of fish genome editing.

Fish	Gene editing nuclease			Alleles generated by		Heritable	References
	ZFN	TALEN	CRISPR	NHEJ	HDR		
Zebrafish	√	√	√	√	√	√	[10,13,20]
Medaka	√	√	√	√	√	√	[26–28]
Tilapia		√	√	√		√	[29,30]
Yellow catfish	√	√		√		√	[32,33]
Channel catfish	√			√			[37]
Southern catfish			√	√			[35]
Common carp		√	√	√			[36]
Rainbow trout	√			√		√	[31]
Atlantic salmon			√	√			[34]
Cavefish		√		√			[40]
Killifish	√		√	√	√		[38,39]
Rohu			√		√		[41]

expression profiles of a large number of genes with conserved function in vertebrate sex determination pathway have been well studied in normal or hormone induced sex reversed tilapia gonad [63–66]. Much valuable information was mainly derived from these expression data. But functional studies in tilapia are difficult to implement partially due to the lack of effective *in vivo* gene manipulation methods, thus hindering studies on their roles in sex determination. Transgenic overexpression of Dmrt1, Foxl2, and their dominant-negative mutants in genetic females and males, respectively, has been performed [64,65]. However, this method was restricted by the low efficiency and specificity.

In recent years, targeted mutagenesis in tilapia has been successfully performed using TALENs and CRISPR/Cas9. Site specific modification was accomplished by injecting one cell stage embryos with a pair of mRNAs encoding TALENs, or with CRISPR/Cas9 components designed to target the genomic DNA. Nuclease activity and mutants were evaluated by T7 endonuclease I (T7EI), restriction enzyme digestion and Sanger sequencing. Several genes, i.e., *foxl2*, *dmrt1*, *cyp19a1a*, *dmrt6*, *amhy*, *amhrII*, *gsdf*, *cyp26a1*, *aldh1a2*, *sf-1* and *igf3*, have been mutated with extremely efficiencies as high as 95% in the F_0 generation [29,30,67–70]. The F_0 generation could be used for analyzing the phenotypic of target gene because of the high efficiency and biallelic mutation induced by the TALEN and CRISPR/Cas9 technologies in tilapia. Our data showed that transcription factor Foxl2 deficiency in F_0 XX fish led to female to male sex reversal in the F_0 generation, further confirming the essential role of Foxl2 in female sex determination [29]. High mutation of Cyp26a1 resulted in advanced meiotic initiation in F_0 XX tilapia and also in southern catfish [68]. If no obvious phenotype was observed in the F_0 generation, the mosaic founder was raised to adulthood as founders to produce F_1, which are then raised to produce F_2 via intercross of heterozygous F_1 fish. Currently, several knockout lines have been established in tilapia including *gsdf*, *amhy* and *cyp19a1a*. Only partial sex reversal (ovotestis) was observed in Cyp19a1a deficiency F_0 XX fish [29], however, complete female to male sex reversal occurs in F_2 individuals and the sex-reversed male was functional (Unpublished data). Phenotypes of TALEN and CRISPR/Cas9 induced gene mutation on gonad development were listed in Table 2. These data suggest that TALENs and CRISPR/Cas9 are available and effective tools for reverse genetics studies in farmed fish and this platform will increase our knowledge about the mechanisms of tilapia sex determination and differentiation. In addition, it is useful to establish conditional knockout platform based on gene editing nuclease in tilapia, which permits more precise dissection of gene function.

4.2. Genetic factors involved in tilapia sex determination

4.2.1. Male sex determination

Master sex determining (SD) genes are the primary genetic switches controlling the gonadal sex differentiation cascade tightly leading to either ovaries or testes. Teleosts form attractive models to study the evolution of sex chromosomes and sex determination systems. In 2002, *dmy*, a *dmrt1* homolog on the Y chromosome, was the first SD gene identified in teleost fish, medaka [74]. Over the past 14 years, several master SD genes have been identified and demonstrated in only a few species (Table 3). anti-Müllerian hormone (Amh) is required for the regression of Müllerian ducts in mammals, birds, and reptiles [75,76]. Both *amh* and its the type II amh receptor (*amhrII*) has been isolated in several fish species despite the fact that they lack Müllerian ducts [66,77]. Recently, the master SD gene *amhy,* a duplicate of *amh*, has been successfully identified in Nile tilapia by our group. By sequencing and assembling the fosmid isolated by sex specific marker (marker-5), two *amh* was tandemly located on the Y chromosome. The former was designated as *amhΔ-y* because there is a 5 bp insertion in the exonVI that produce a truncated protein. The latter was named as *amhy* and the open reading frame of *amhy* was identical to the *amh* on the X chromosome except for a missense SNP (C/T) changing an amino acid (Ser/Leu92). In addition, there is also another difference that a large fragment was lost in the promoter of *amhy*. Transcriptome analysis showed that both *amhy* and *amhΔ-y* expression are exclusively expressed in the XY gonads from 5 dah onwards. These data cannot demonstrate which the SD gene is. The strong evidences to support the *amhy* as SD gene are obtained via loss of these two *amh* in XY fish. As expected, knockout of *amhy* in XY tilapia led to male to female sex reversal, while knockout of *amhΔ-y* not by using CRISPR/Cas9. In agreement with male to female sex reversal, the Cyp19a1a, the gene encoding the aromatase that converts androgen to estrogen, was expressed in the sex-reversed XY gonads at 10 dah and finally the correspond serum estrogen (E2) increases in adult, which was similar to the control ovary. In contrast, male specific gene Dmrt1 was lost similar to those control ovaries. Furthermore, loss of *amhrII* in XY fish also resulted in male to female sex reversal [73]. Therefore, *amhy*/*amhrII* is essential for male sex determination in tilapia, as demonstrated by loss of function studies using CRISPR/Cas9.

The teleost specific TGF-β gene *gsdf* was newly identified as another key factor involving in male sex differentiation and maintenance in tilapia. In the XY *gsdf* knockout fish, the gonads first only expressed Dmrt1 protein suggesting the initiation of male pathway

Table 2
Phenotypes of TALEN and CRISPR/Cas9 induced gonadal related gene mutation in tilapia.

Gene	Methods	Generation	Genotype	Phenotype	Refs.
dmrt1	TALEN	F_0	XY	Disruption of spermatogenesis	[29]
foxl2	TALEN	F_0	XX	Female to male sex reversal (testis)	[29]
		F_2	XX	Female to male sex reversal (testis)	Unpublished
cyp19a1a	TALEN	F_0	XX	Partial female to male sex reversal (ovotestis)	[29]
		F_2	XX	Female to male sex reversal (testis)	Unpublished
β-*catenin1/2*	TALEN	F_0	XX	Retardation of ovarian differentiation and masculinization	[71]
R-spondin1	TALEN	F_0	XX	Retardation of ovarian differentiation and masculinization	[72]
			XY	Delay in spermatogenesis	[72]
amhy	CRISPR/Cas9	F_0	XY	Male to female sex reversal (ovary)	[73]
	CRISPR/Cas9	F_1/F_2	XY	Male to female sex reversal (ovary)	[73]
amhrII	CRISPR/Cas9	F_0	XY	Male to female sex reversal (ovary)	[73]
gsdf	CRISPR/Cas9	F_0	XY	Partial to complete male to female sex reversal	[70]
	CRISPR/Cas9	F_2	XY	Male to female sex reversal (ovary)	[70]
sf-1	CRISPR/Cas9	F_0	XX/XY	Gonadal dysgenesis	[69]
		F_1	XX	Sex reversal and fertile males	[69]
dmrt6	CRISPR/Cas9	F_0	XY	Disruption of spermatogenesis	[67]
aldh1a2	CRISPR/Cas9	F_0	XX	Delay in meiosis initiation	[68]
cyp26a1	CRISPR/Cas9	F_0	XY	Advanced meiosis	[68]

Table 3
An overview of master sex determining gene identified in fish.

Fish species	SD system	SD gene	Gene family	Methods	Year	Refs.
Medaka (*Oryzias latipes*)	XX/XY	*dmy*	DM-domain family gene	cDNA overexpression; TILLING	2002	[74]
Half-smooth tongue sole (*Cynoglossus semilaevis*)	ZZ/ZW	*dmrt1*	(Transcription factor)	–	2014	[8]
Medaka (*Oryzias luzonensis*)	XX/XY	$gsdf^{Y}$	TGF-β signal pathway	fosmid overexpression	2012	[82]
Patagonian pejerrey (*Odontesthes hatcheri*)	XX/XY	*amhy*	(Growth factor)	morpholino	2012	[81]
Fugu (*Takifugu rubripes*)	XX/XY	*amhrII*		–	2012	[84]
Tilapia (*Oreochromis niloticus*)	XX/XY	*amhy*		CRISPR/Cas9;fosmid and cDNA overexpression	2015	[73]
Killifish (*Nothobranchius furzeri*)	XX/XY	$gdf6^{Y}$		–	2016	[83]
Medaka (*Oryzias dancena*)	XX/XY	$sox3^{Y}$	Sox family gene (Transcription factor))	ZFN; BAC overexpression	2014	[79]
Rainbow trout (*Oncorhynchus mykiss*)	XX/XY	*irf9*	Immune-related gene	ZFN	2012	[85]

at 10 dah. Then simultaneous expression of Dmrt1 and Cyp19a1a at 18 dah was observed implying both male and female pathways were activated. A shift to the female pathway expressing only Cyp19a1a at 36 dah follow next, and finally the gonads developed into ovaries (Fig. 2) [70]. Consistently, overexpression of Gsdf in XX fish resulted in sex reversal and exhibited typical testis structure and normal spermatogenesis [78]. Therefore, Gsdf is a downstream factor involved in the male sex determination pathway of tilapia. In two medaka species, *gsdf* expression was also directly regulated by the SD genes (*dmy* and $sox3^{Y}$) to initiate testicular differentiation. Further experiment provide strong evidences that loss of Gsdf in XY medaka and gain of Gsdf in XX medaka resulted in sex reversal, suggesting Gsdf might be a well conversed factor implicated in the downstream of SD pathway in teleosts [42,79].

It is worthy to note that a missense SNP mutation in the kinase domain of medaka AmhrII (hotei mutant) resulted in male to female sex reversal in half of XY fish even though the SD gene is *dmy* in this species [80]. It has also reported that four other SD genes in fishes also belong to TGF-β family members including the *amhy* in *Patagonian pejerrey*, *amhrII* in *Takifugu rubripes*, $gsdf^{Y}$ in *Oryzias luzonensis* and $gdf6^{Y}$ in Killifish [81–84]. Among the nine SD genes identified so far, half of which belong to TGF-β family members (Table 3). Taken together, these findings highlight a critical role for TGF-β signaling pathway in teleost sex determination. A fundamental question in biology is how sex is determined. It is well accepted that the PGC number and estrogen level could affect sex differentiation in fish species. The elucidation of how TGF-β signaling pathway control gonadal fate, such as regulating germ cell proliferation/apoptosis and estrogen production, would lead to a better understanding of sex determination in fish.

4.2.2. Female sex determination

The transcription factor Foxl2 is involved in vertebrate female sex determination. Our previous studies demonstrated that Foxl2 plays a decisive role in the ovarian differentiation of the tilapia by regulating aromatase/Cyp19a1a expression. Although the XY fish with wild type Foxl2 overexpression never display complete sex reversal, overexpression of foxl2 dominant negative mutant in XX fish induces varying degrees of testicular development and decreased serum estrogen levels [64]. Consistently, Foxl2 and its target gene Cyp19a1a deficiency in XX fish lead to female to male sex reversal in F_0 XX fish, further confirming the essential role of Foxl2 and estrogen in female sex determination [29]. Homozygous mutation of foxl2 in XX tilapia also resulted in sex reversal [Unpub-

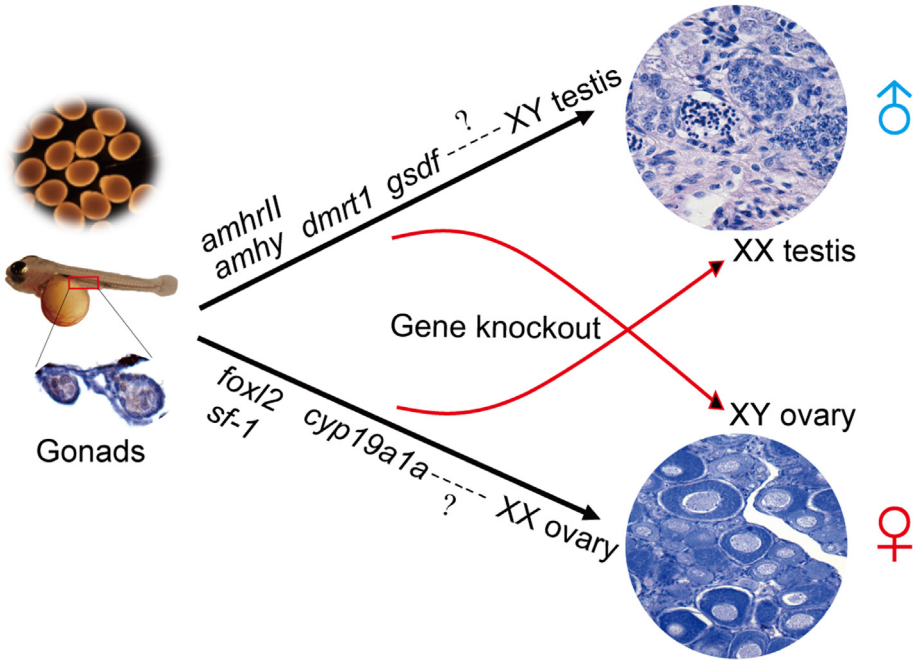

Fig. 2. (Color online) Genes identified in tilapia sex determination pathway. Loss of *amhy*, *amhrII*, *gsdf* or *dmrt1* in XY fish induces ovarian development, while gain of function of these genes in XX fish results in testicular development. In contrast, knockout of *foxl2*, *sf-1* or *cyp19a1a* in XX fish induces testicular development, while overexpression of *foxl2* or *cyp19a1a* in XY fish results in ovarian development.

lished data]. The germ cell specific *foxl3*, a paralog of *foxl2*, was also involved in medaka female sex differentiation. Disrupted of *foxl3* in XX medaka resulted in ovotestis which could produce functional sperm and eggs simultaneously [86]. In addition, heterozygous mutation of *sf-1* in XX tilapia also resulted in female to male sex reversal (Fig. 2) [69]. These results demonstrate that Foxl2, Sf-1 and Cyp19a1a play key role in tilapia female sex determination. In addition, gonadotropins, *fshb*, and *lhb*, and their receptors, *fshr* and *lhr* were also demonstrated to act on fish sex differentiation and maintenance by TALEN. In zebrafish and medaka, mutation of *fshr* deficient females exhibited failure of oocytes development and all females reversed to fertile males [87,88]. Double mutation of *fshb:lhb* or *fshr:lhr* resulted in all male progeny [89]. These results suggested that gonadotropins play key roles in female gonadal sex differentiation and maintenance. The zebrafish with *dax1* or *fancl* mutation develop as all fertile males [90,91]. In mammals, R-spondin1, an activator of Wnt/β-catenin signaling pathway, is located upstream of the female sex determination pathway. Knockdown of R-spondin1 or its downstream factor β-catenin resulted in retardation of ovarian differentiation and masculinization of the gonads [71,72]. In contrast, ectopic overexpression of R-spondin1 induced femininity in XY medaka [92]. In all, the genes identified in female sex determining pathway were listed in Table 4. These data suggested that TALENs and CRISPR/Cas9 are critical for understanding gene functions in fish. These techniques will facilitate the identification of new factors regulating sex determination in the future.

MicroRNAs (miRNAs) are well conserved small noncoding RNAs that silence gene expression post transcriptionally by binding to 3′ untranslated regions of mRNAs. miRNAs participate in various biological processes, including gonad development and sex differentiation. Recently, 635 mature miRNAs were identified in 5 dah tilapia gonads, the critical stage for sex determination. Further analysis showed that 49 and 62 miRNAs had higher expression in XY and XX gonads, respectively [63]. Successful disruption of miRNAs has been reported in zebrafish [93]. Therefore, it is now possible to decipher the roles of these differentially expressed miRNA in fish sex determination, which will add new information to the regulatory network of molecular sex determination in teleosts. In all, the knowledge of the genetic cascade of sex determination is useful for sex control and for the implementation of breeding programs for aquaculture.

4.2.3. Production of fast growing tilapia

Sexual dimorphism in body growth is a common phenomenon in fish [94]. It is well known that a large number of fish species display sexual dimorphism in growth rates [94]. For example, female half-smooth tongue sole grow faster than males, whereas male tilapia grow faster than females [8,61]. Sex control is important for aquaculture owing to economics. Mono-sex culturing could avoid unwanted reproduction. In aquaculture, male tilapia monosex populations are obtained by androgen treatments due to the limited availability of YY supermales. Hormone treatment has raised concerns because of the possible effects of hormone residues on water quality and biodiversity. Establishment of a sex control alternative based on nonhazardous approach represents a major challenge for aquaculture. The best way to obtain mono-sex populations is through genetic control [95]. So far, one effective method for producing genetically male tilapia on a large scale is to cross normal XX females with YY supermale broodstock [96]. Recently, sex-linked DNA markers have been successfully isolated and marker-assisted selection system has been established in Nile tilapia [62]. Although many YY supermales could be obtained by crossing XY females with YY males in laboratory, it is difficult to obtain all YY populations because the fertility of YY females is partially impaired in our strain (Unpublished data). Therefore, this constrains the wide applications of this method in aquaculture. Further studies need to explore the possibility of producing fertile YY females by using TALENs and CRISPR/Cas9 (Fig. 3). Therefore, the establishment of efficient genome editing method in tilapia will greatly promote genetic engineering and sex control in aquaculture.

It has been reported that in Mozambique tilapia gonads directly regulate body growth, implying some genes expressed in gonads also participate in controlling growth [97,98]. It is well known that *sf-1* knockout mice were significantly heavier by 8 weeks of age and weighed almost twice as much as wild type controls. Obesity in *sf-1* knockout mice predominantly resulted from decreased activity rather than increased food intake [99]. Recently, we reported that the *sf-1* heterozygous mutant tilapia displayed heavier body weight and higher condition factor compared with the control even though their body length was indistinguishable. This might be caused by decreased aggressive behavior and decreased investment in reproduction, as reflected by the low estrogen and androgen levels and small gonads of the Sf-1 deficient fish [69]. Elucidation of the molecular mechanisms involved in this process might contribute to the development of fast growing strains for aquaculture. In addition, it is important to investigate whether the sex-reversed XX males showed faster growth than XX females, which might also be a choice of producing fast growing tilapia.

Genetic engineering of important genes that control body growth and size is a most promising method for obtaining fast growing fish in the future. One candidate gene is *myostatin* (*mstn*), a negative regulator of muscle growth. Mammals including mice, cattle, sheep, dog, and mouse with spontaneous mutations in their *Mstn* genes all exhibited double muscle phenotype [100,101]. Similar to mammals, knockout or repression of *mstn* in medaka and zebrafish also yields a larger phenotype [102,103]. In addition, zebrafish with deficient *socs2* displayed higher growth rate than the

Table 4
Genes involved in female sex determining pathway in fish.

Fish	Gene	Method	Genotype	Phenotype	Refs.
Tilapia	*foxl2*	cDNA overexpression	XY	Sex reversal	[64]
		TALEN	XX	Sex reversal	[29]
	cyp19a1a	TALEN			[29]
	sf-1	CRISPR/Cas9			[69]
Zebrafish	*dax1*	TALEN	Not identified	Sex reversal	[90]
	fancl	Tol2 insertion			[91]
	fshr	TALEN		Sex reversal	[87]
	fshb;lhb	TALEN		All male progeny	[89]
	fshr;lhr	TALEN			[89]
Medaka	*fshr*	TILLING	XX	Sex reversal in some cases	[88]
	foxl3	ZFN		Partial sex reversal	[86]
	r-spondin1	cDNA overexpression	XY	Sex reversal	[92]

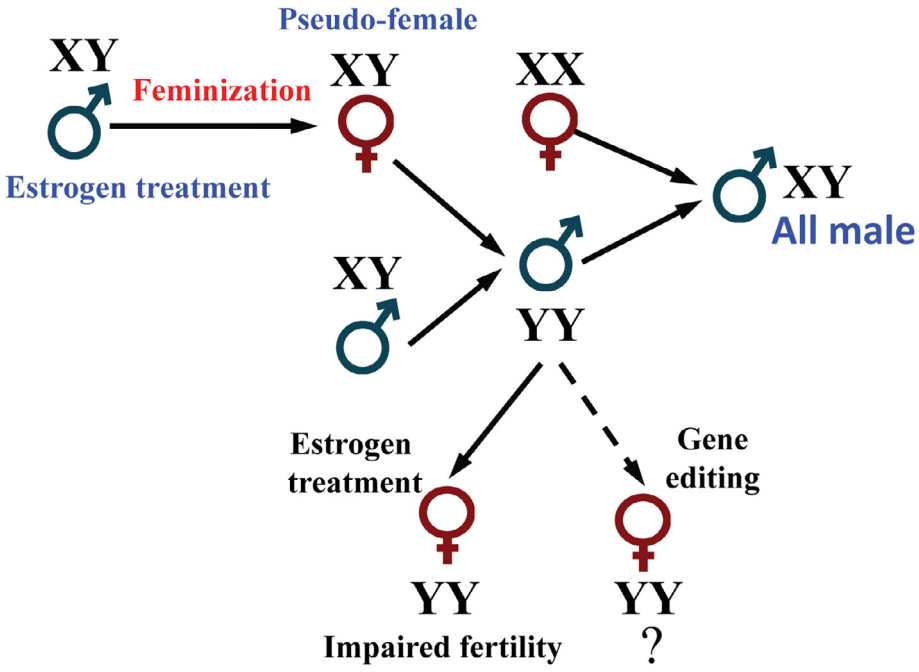

Fig. 3. (Color online) Schematic diagram of the production of all XY populations. The XY fish can be sex reversed to females with estrogen treatment during 5–35 dah. The YY supermale can be produced via intercross of XY pseudo-female with wild type XY male, in which about 1/4 YY supermale can be isolated by sex specific marker-5. All XY fish were obtained by crossing a wild type female (XX) with a supermale (YY). Although YY female was obtained by estrogen treatment, the fertility of YY female was impaired and cannot produce normal eggs. It is possible to produce fertile YY females by using TALENs and CRISPR/Cas9.

control group [104]. Lastly, other growth related factors, such as growth hormone (GH), insulin-like growth factor-1 (*igf-1*), also play important roles in animal growth [105,106]. Therefore, it is necessary to explore whether knockout of these genes in tilapia and also other fishes will produce higher growth rates in the future.

4.2.4. Production of transgenic infertile tilapia

Generating triploid fish is the most common strategy adopted to produce sterile fish. However, the growth rate of triploid tilapia was slow compared with diploids [107]. Recently, an F_0 sterile transgenic tilapia and common carp was generated through expression of antisense RNA to block the endogenous gonadotropin-releasing hormone (GnRH) [108,109]. We reported that knockout of primordial germ cells (PGCs) related *nanos2* and *nanos3* in tilapia using CRISPR/Cas9 produce infertile individuals in F_0 generation [30]. Unfortunately, the sterile trait in these F_0 founders is not heritable. The *dead end* (*dnd*) is a key factor essential for PGCs migration and survival. Knocked down of *dnd* resulted in disruption of PGCs migration and then the apoptosis of PGCs occurs. It has been reported that knockout or knockdown of *dnd* in several fishes resulted in infertile individuals. Recently, it is reported that a strategy for fish that renders the offspring sterile but leaves their parents fertile by using Gal4/UAS for controlling *dnd* expression [110]. Therefore, further studies are needed to establish two transgenic lines, which have Gal4 and UAS driving Cas9 and *dnd* target gRNA, respectively, to obtain infertile offspring.

5. Conclusion

To meet the challenges in aquaculture and produce genetically modified fishes, it is critical to improve both basic and applied research in fish biology. The establishment of TALENs and CRISPR/Cas9 technologies in tilapia opens up new doors for understanding basic biology, and provides powerful tools for investigating traits important for the aquaculture.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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