General and Comparative Endocrinology 277 (2019) 9–16

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Roles of estrogens in fish sexual plasticity and sex differentiation

Minghui Li, Lina Sun, Deshou Wang*

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Sciences, Southwest University, 400715 Chongqing, PR China

ARTICLE INFO

Keywords:
Estrogen
Ovarian differentiation
Sexual plasticity
Sex control
Fish

ABSTRACT

Fish sex could be reversed at the undifferentiated stage of gonad by administration of exogenous estrogen (E2) or blockade of endogenous estrogen synthesis with aromatase inhibitors, which is designated as primary sex reversal (PSR). Recent studies have well demonstrated that gonochoristic fish maintain their sexual plasticity after sex determination/differentiation. The differentiated ovary could be transdifferentiated into functional testis, and vice versa, the differentiated testis could be transdifferentiated into ovary. By analyzing these two secondary sex reversal (SSR) models, it was found that induction of male-to-female sex reversal initiates from dorsal (near the blood vessel) to the ventral, while induction of female-to-male sex reversal initiates from the ventral to dorsal. Down regulation of endogenous estrogen is the prerequisite for the ovarian transdifferentiation. However, exogenous estrogen alone is not sufficient for inducing differentiated testis to ovary. Administration of E2 and simultaneous blockage of androgen synthesis could induce testicular transdifferentiation. Therefore, endogenous estrogen is critical for the ovarian differentiation/maintenance and androgen is critical for testicular maintenance. Recently, genetic studies with genome editing technologies also showed that disruption of Cyp19a1a induced testicular development, indicating that *cyp19a1a* is the key gene essential for estrogen synthesis and ovary differentiation/maintenance. Knockout of male pathway genes or overexpression of female pathway genes could up-regulate *cyp19a1a* expression and increase estrogen level so as to promote ovary. Conversely, knockout of female pathway genes or overexpression of male pathway genes could down-regulate *cyp19a1a* expression and decrease estrogen level so as to promote testis (transgenic or knockout sex reversal, TSR). Epigenetic regulation of *cyp19a1a* play a critical role in natural sex reversal (NSR), but its relation with PSR, SSR and TSR needs further detailed investigations. In all, these studies further highlighted the important roles of endogenous estrogens in fish sex differentiation/maintenance.

1. Introduction

Sex is a common phenomenon in the animal kingdom. Sexual dimorphism in growth rate and body size is popular in fish species (Mei and Gui, 2015). Fish sex determination and differentiation is an attractive issue because sex control has great commercial interest in aquaculture (Devlin and Nagahama, 2002). Fish sex is determined either by genetic factors or environmental factors, or a combination of both. Environmental factors, especially sex steroid hormones, could even override the genetic factors and determine gonad fate (environmental dependent sex determination, ESD) (Nagahama, 2002). Estrogens are produced by the conversion of androgens through cytochrome P450 aromatase, which was mainly encoded by *cyp19a1a/b* in fish (Simpson et al., 1994; Zhang et al., 2014). In teleosts, aromatase and endogenous estrogens are specifically expressed and synthesized in the female gonads during the critical period of molecular sex differentiation and act as natural inducer of ovarian differentiation (Nagahama, 2000; Tao et al., 2013). Previous studies in several fish species demonstrated that female-to-male sex reversal is associated with a decrease in estrogen levels, while male-to-female sex reversal is associated with an increase in estrogen levels (Guiguen et al. 1993, 1999; Chang et al., 1994; Piferrer et al., 1994; Piferrer, 2001; Tsai et al., 2011; Murata et al., 2014). Therefore, estrogens are essential factors for inducing ovarian development in fish. The roles of estrogens in fish gonadal sex

Abbreviations: ESD, environmental dependent sex determination; NSR, natural sex reversal; AI, aromatase inhibitor; MT, 17α-methyltestosterone; PSR, primary sex reversal; SSR, secondary sex reversal; TR, trilostane; MN, metopirone; GA, glycyrrhetinic acid; ZFNs, zinc-finger nucleases; TALENs, transcription activator-like effector nucleases; CRISPR/Cas9, clustered, regularly interspaced, short palindromic repeats/CRISPR-associated systems; ENU, N-ethyl-N-nitrosourea; E2, estradiol-17β

* Corresponding author.
E-mail address: wdeshou@swu.edu.cn (D. Wang).

https://doi.org/10.1016/j.ygcen.2018.11.015
Received 1 October 2018; Received in revised form 23 November 2018; Accepted 26 November 2018
Available online 27 November 2018
0016-6480/ © 2018 Published by Elsevier Inc.

differentiation were well reviewed and summarized previously (Guiguen et al., 2010). It was concluded that Cyp19a1a is not only important for ovarian, but also for testicular differentiation in both gonochoristic and hermaphrodite fish species. This review summarizes new data on the studies of estrogens in fish sex differentiation and sexual plasticity from transcriptome, epigenetics, chemical treatment, and especially gene knockout analyses. We concluded that endogenous estrogens are important for fish ovarian differentiation and maintenance.

2. Estrogen is a key factor involved in fish sex differentiation and maintenance

2.1. Natural sex reversal (NSR)

Natural sex reversal (NSR) occurs in more than 300 species (hermaphrodites, either protandrous or protogynous). Hermaphroditic fish have a plastic sex and exhibited natural sex reversal during their life spans. Although the underlying mechanisms for this sex reversal remain unclear, the majority of studies have focused on the roles of estrogens during this process (Chang et al., 1995; Wu et al., 2008, 2016; Zhang et al., 2013; Murata et al., 2014; Li and Wang, 2017). In protandrous fish, previous studies have shown that increase of endogenous estrogen level was associated with male-to-female sex reversal, such as Asian seabass (*Lates calcarifer*) and black porgy (*Acanthopagrus schlegeli*) (Guiguen et al. 1993; Chang et al., 1994). For example, the black porgy are functional males for the first two years of life but begin to sexually change to females during the third year (Chang and Yueh, 1990). During NSR, *cyp19a1a* expression was found to be significantly up-regulated in interstitial cells of the testicular tissues. Blockage of aromatase activity by its inhibitor Fadrozole (AI) is able to disrupt NSR (Wu et al., 2008). In black porgy younger than 2 years old, exogenous estradiol-17β (E2) can induce ovarian development with only primary oocytes. The induced ovary can be maintained during the period of E2 treatment. However, the induced ovary will developed into complete testis spontaneously after E2 treatment was terminated (Fig. 1A). Although the gonads developed as ovary, gene expression analyses showed that *cyp19a1a* was not up-regulated to the corresponding level as that in the wild type ovary (Wu et al., 2016), suggesting endogenous estrogen was not synthesized to maintain ovary. This indicates that the endogenous estrogens were essential for ovarian maintenance.

In protogynous fish, E2 level was found to be decreased during female-to-male natural sex change in several fish species, such as grouper (*Epinephelus akaara*) and ricefield eel (*Monopterus albus*) (Huang et al., 2009; Zhang et al., 2007). Exogenous 17α-methyltestosterone (MT) or AI could induce testicular development in two grouper species, malabar grouper (*Epinephelus malabaricus*) and orange-spotted grouper (*Epinephelus coioides*), which first developed as females and then some of the grouper could be sex reversed to males (Bhandari et al., 2004, 2006; Wu et al., 2015; Wang et al., 2018). The induced testes were maintained during continuous MT or AI treatment (Tsai et al., 2011; Murata et al., 2014). However, the male sex will reverse to female after MT or AI treatment was withdrawn. Gene expression analysis showed that no apparent differences were observed in the expression patterns of steroidogenic enzymes between the MT-induced mature testes and the control immature ovaries. In addition, the level of serum E2 level in MT or AI-treated fish was the same as those in control female fish (Murata et al., 2014; Wu et al., 2015), indicating that endogenous estrogen level was not down-regulated in grouper although female-to-male sex change occurs. Therefore, once the exogenous MT treatment was terminated, the sex of protogynous fish will reverse back to females. However, a recent study on the orange-spotted grouper showed that the MT induced male sex can be maintained after MT feeding withdrawal if the gonads contain only male germ cells (Wang et al., 2017). It was found that MT feeding induced sex reversal can be divided into early and late phases. In the early phase with both male and female germ cells, the gonads will reverse back to the ovaries after MT feeding withdrawal. In the late phase with only male germ cells, the induced testes could be maintained even though MT-feeding is withdrawn (Fig. 1B). *cyp19a1a* mRNA was markedly increased after MT withdrawal in both phases. However, E2 levels were increased in the early but not in the late phase, which explained why early MT-induced male fate could be further reversed back to ovary. Based on these results, it can be concluded that endogenous estrogen should be induced to maintain ovarian phenotype and conversely it should be repressed to maintain testicular phenotype in protogynous fish.

In recent years, epigenetic modification has been found to contribute to the regulation of *cyp19a1a* expression during NSR. In black porgy, male-to-female sex change occurs followed by decrease of *cyp19a1a* promoter methylation level. However, *cyp19a1a* promoter was also hyper-methylated in the exogenous E2 induced ovary, which might explain why Cyp19a1a and endogenous estrogen level was not up-regulated. Further investigation revealed that the methylation of the *cyp19a1a* promoter was significantly decreased in the ovary tissue after the testis was removed from the digonic gonad and the fish changed sex from male-to-female (Wu et al., 2016; Wu and Chang, 2017), suggesting some factors originated from testis could influence the level of *cyp19a1a* promoter methylation. In ricefield eel, *cyp19a1a* promoter

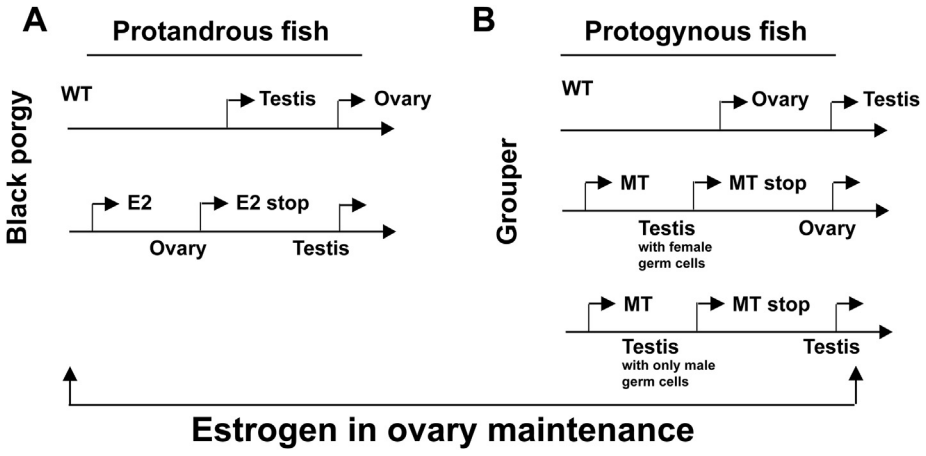

Fig. 1. Exogenous steroid hormone induced sex reversal in hermaphroditic fish. (A) In protandrous black porgy, they are males for the first two years but change to females during the third year. Exogenous estradiol-17β (E2) can induce ovarian development with only primary oocytes for the first two years. The induced ovary will developed into complete testis spontaneously after E2 treatment was terminated because endogenous E2 was not upregulated. (B) In protogynous orange-spotted grouper, they first developed as females at the age of 4–5 years and then some of the grouper could be sex reversed to males. Exogenous 17α-methyltestosterone (MT) or AI could induce testicular development, which will be reversed to female after MT or AI treatment was withdrawn if the gonads have both male and female germ cells. Endogenous estrogen level was not down-regulated in grouper although female-to-male sex change occurs. However, MT induced male sex can be maintained after MT feeding withdrawal if the gonads contain only male germ cells.

was hypermethylated in the ovotestis and testis compared with that in the ovary. DNA methylation level of *cyp19a1a* promoter in the gonads was increased during sex change from female to male. Importantly, the DNA methylation inhibitor treatment could reverse the natural female to male sex change (Zhang et al., 2013). These results suggested that epigenetic control of *cyp19a1a* gene expression play an important role in the natural sex change in teleost. Epigenetic regulation was also proposed to be involved in ESD species in response to environmental influences, leading to the gonad fate change (Manolakou et al. 2006). Exposure to high temperature, the methylation level of *cyp19a1a* promoter was significantly increased during the early development stage leading to suppressed *cyp19a1a* expression and male development in European sea bass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2009; Navarro-Martín et al., 2011). High temperature treatment resulted in up-methylation of *cyp19a1a* promoter and led to testicular development in ZW half-smooth tongue sole (*Cynoglossus semilaevis*) (Shao et al., 2014). These data indicated that the level of endogenous estrogen is tightly controlled by epigenetic modification of *cyp19a1a* promoter and is important for NSR.

2.2. Primary sex reversal (PSR)

Most teleosts are gonochorists, which develop either as males or females, and do not change sex throughout their life spans. Treatment with steroid hormones prior to sexual differentiation has been shown to induce ovary or testis development according to the type of steroids administered, which is designated as artificial primary sex reversal (PSR). Among various hormones, E2 and AI are commonly used inducers for PSR. Administration of exogenous E2 can reverse genetic males to phenotypic females in teleosts if the treatment is applied before or during the narrow window of sex differentiation (Piferrer et al., 1994; Guiguen et al., 1999; Piferrer, 2001; Afonso et al., 2001; Kwon et al., 2001; Vizziano et al., 2008). In contrast, administration of genetic females with AI that block estrogen synthesis can induce phenotypic males when applied during the critical period of sex differentiation in a number of fish species (Kitano et al., 1999; McAllister and Kime, 2003; Fenske and Segner, 2004; Uchida et al., 2004; Komatsu et al., 2006; Iwamatsu et al., 2006). Our recent study showed that simultaneous treatment of XX and XY tilapia (*Oreochromis niloticus*) with both androgen (MT) and E2 before sexual differentiation resulted in development of all females, supporting the conclusion that estrogen could maintain the female phenotype of XX fish and feminize XY fish even in the presence of androgen, while androgen cannot induce sex reversal of XX fish in the presence of estrogen (Chen et al., 2016) (Fig. 2A). These

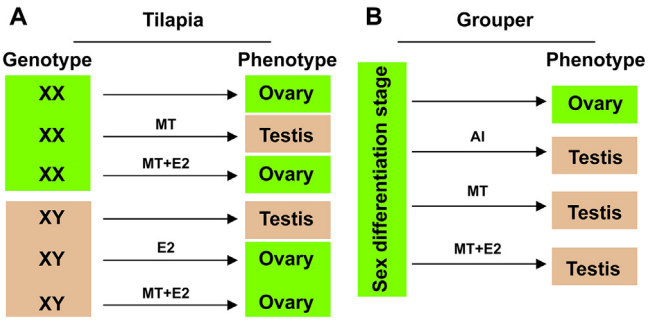

Fig. 2. Effects of administration of both estrogen and androgen on fish gonadal differentiation. (A) In gonochoristic tilapia, treatment of XX tilapia with 17α-methyltestosterone MT induced testicular development and treatment of XY tilapia with estradiol-17β(E2) induced ovarian development before gonad differentiation. Simultaneous treatment of XX and XY fish with both MT and E2 before sexual differentiation resulted in all females. (B) In hermaphroditic orange-spotted grouper, treatment with aromatase inhibitor (AI) or MT induced testicular development. In contrast to tilapia, treatment of grouper with both MT and E2 masculinize the gonads.

results further highlight the importance of estrogens in sex differentiation. It is worth mentioning that, in contrast to our results, recent study in protogynous orange-spotted grouper showed exogenous androgen can masculinize the gonads even in the presence of an equal amount of estrogen (Huang et al., 2018) (Fig. 2B). It is interesting to investigate whether the gonads will develop as ovary with both estrogen and androgen administration in protandrous fish.

Different from sex reversal induced by exogenous hormone in hermaphrodite fish, the reversed sex could be maintained in gonochorists even though the exogenous E2 or AI treatment was terminated. Detailed analysis revealed that exogenous estrogen or AI treatment resulted in significant up or down regulation of steroidogenic enzymes, especially the Cyp19a1a, for endogenous estrogen synthesis in PSR (Chen et al., 2016). The up and down regulation of endogenous estrogen level contribute to PSR and maintain the sex finally.

2.3. Secondary sex reversal (SSR)

Although sex reversal could be induced by sex hormone, most vertebrates were believed to have lost sexual plasticity after the terminal differentiation of their gonads and remain the same sex throughout their life spans. Once the fish grows beyond the stage, artificial inducement of sex reversal is difficult or even impossible. Therefore, gonochoristic fish was thought to be completely lose their sexual plasticity after terminal differentiation of the gonad into either ovary or testis. Three recent reports demonstrate that complete sex reversal, designated as artificially induced secondary sex reversal (SSR), could be induced by down-regulation of endogenous estrogens with long-term AI treatment in adult females of tilapia, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) (Paul-Prasanth et al., 2013; Takatsu et al., 2013; Sun et al., 2014). The sex-reversed male fish showed normal secondary sexual characteristics and produced fertile sperm after termination of AI administration. These results suggested that gonochoristic female fish still maintain their sexual plasticity until adulthood. Constant endogenous estrogen production is needed for ovarian phenotype maintenance during all life span. However, the threshold of estrogen level for maintaining ovarian phenotype is still unknown.

Induction of sex reversal of male fish has been restricted to the sex undifferentiated period. We found that exogenous E2 alone treatment could not induce male-to-female SSR in one-month-old tilapia. Transcriptome analysis revealed that steroidogenic enzymes, especially Cyp11b2 encoding the key enzyme for androgen synthesis, were expressed in the one-month-old testis, but not in the undifferentiated XY gonads (Tao et al., 2013). Inhibition of androgen synthesis by trilostane (TR), metopirone (MN) and glycyrrhetinic acid (GA) (inhibitor of 3β-HSD, Cyp11b2 and 11β-HSD, respectively) alone also resulted in no secondary sex reversal. However, blockage of androgen synthesis and simultaneous administration of E2 can induce the differentiated testis to transdifferentiate into ovary (Shi et al., 2017). This result demonstrated that androgen plays a critical role in testis maintenance. E2 alone is not sufficient for inducing testicular transdifferentiation. To date, this male-to-female sex reversal can only be achieved in one-month-old tilapia. Why induction of differentiated testis to ovary in gonochoristic fish is so difficult compared with females? The relevance of sex reversal with 11-KT concentration, which is increasing during testis development, needs to be further investigated.

2.4. Transgenic (knockout) sex reversal (TSR)

Although estrogens have been generally considered to play a critical role in ovarian differentiation in non-mammalian vertebrates, our knowledge about their functions was obtained mainly from exogenous hormone or chemical administration. There is a lack of genetic data on the importance of estrogen in fish. This is partly due to the lack of powerful genetic approaches for functional studies in fish. Now, this situation is changed with the recent development of engineered

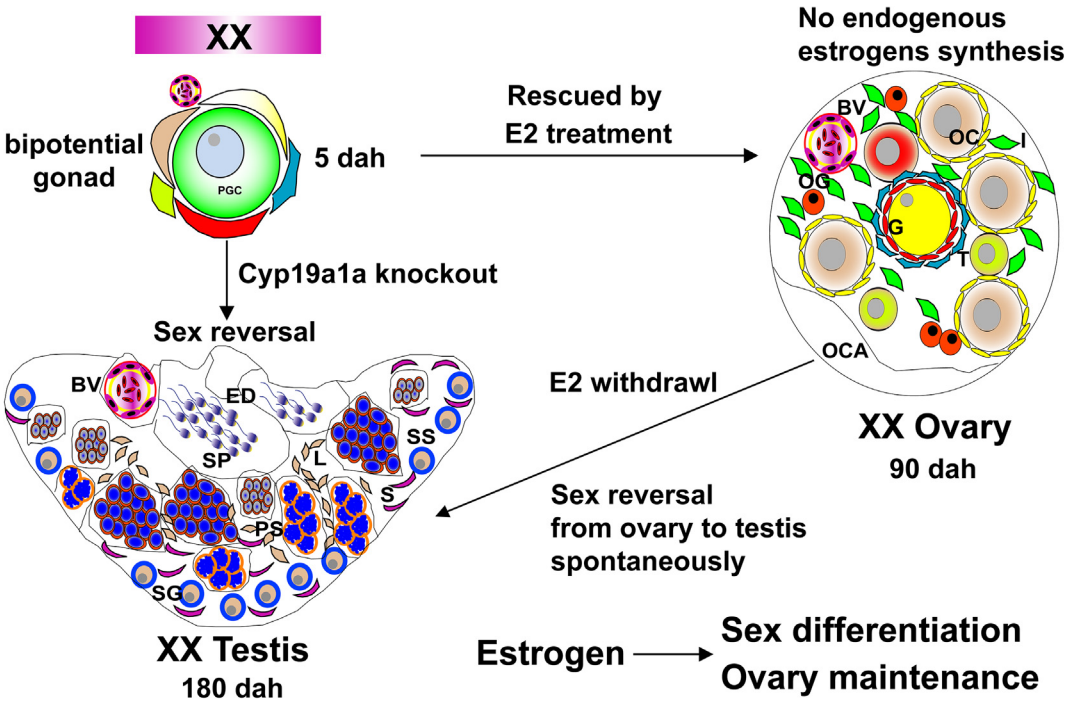

Fig. 3. Cyp19a1a is essential for fish ovary differentiation and maintenance. Knockout of *cyp19a1a* resulted in female to male sex reversal in fish. This sex reversal can be successfully rescued to restore ovary by exogenous estradiol-17β (E2) treatment. However, the ovary of rescued *cyp19a1a* mutants will reverse back to testis after withdrawal of E2 because no endogenous E2 can be synthesized to maintain ovary phenotype. dah, days after hatching. PGC, primordial germ cell. BV, blood vessel. OC, oocyte. G, granulosa cells. OG, oogonia. I, interstital cells. OCA, ovary cavity. T, theca cells. ED, efferent duct. SG, spermatogonia. SS, spermatocyte. SP, sperm. PS, primary spermatocyte. L, leydig cells. S, sertoli cells.

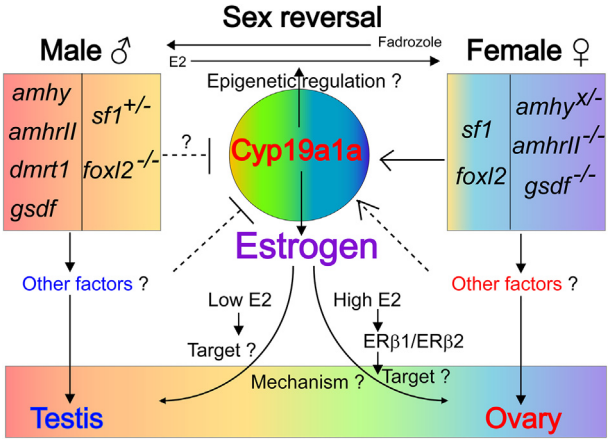

Fig. 4. Endogenous estrogens are important for fish sex differentiation. In tilapia, the male pathway genes could repress *cyp19a1a* expression and decrease estrogen level so as to promote testis formation. In contrast, the female pathway genes up-regulate *cyp19a1a* expression and increase estrogen level so as to promote ovary formation. Estrogens regulate downstream genes by binding to ERβ1/ERβ2. The mechanisms (target and signal pathway) for estrogens action to ovary differentiation/maintenance will be an interesting issue to be addressed in the future.

nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered, regularly interspaced, short palindromic repeats/CRISPR-associated systems (CRISPR/Cas9), which have been used to develop genome editing models precisely in a wide variety of organisms including fishes (Li et al., 2013, 2014; Li and Wang, 2017). Because of the teleost-specific whole genome duplication event, two types of *cyp19a1* genes encoding aromatase, which were termed *cyp19a1a* and *cyp19a1b*, were isolated and characterized in a large number of fish species. AI treatment might inhibit the activity of both types of aromatase. It was therefore an open question whether one or both of these enzymes played a role in female sexual differentiation. The expression of *cyp19a1a*/Cyp19a1a is mainly restricted to the ovary, and *cyp19a1b* is most highly expressed in the brain. Although all the information accumulated points to the importance of Cyp19a1a in fish ovarian differentiation, no direct genetic evidences were provided to support this conclusion.

Until recently, *cyp19a1a* and *cyp19a1b* were successfully mutated by TALEN and CRISPR/Cas9 in zebrafish (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017) and tilapia (Li et al., 2013; Zhang et al., 2017). *cyp19a1a* mutant was also generated in medaka by N-ethyl-N-nitrosourea (ENU) method (Nakamoto et al., 2018). No sex reversal was observed in zebrafish, medaka and tilapia *cyp19a1b* mutants (Dranow et al., 2016; Lau et al., 2016; our unpublished data). In contrast, in XX tilapia, knockout of *cyp19a1a* resulted in female-to-male sex reversal (Li et al., 2013; Zhang et al., 2017). In zebrafish, three groups reported that knockout of *cyp19a1a* or both *cyp19a1a* and *cyp19a1b* also resulted in all male populations (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017). The inconsistency is whether the gonad first experienced an ovarian-like stage or not in the mutants. In XX medaka, gonads first differentiated into normal ovaries containing many ovarian follicles that failed to accumulate yolk. Subsequently, ovarian tissues underwent extensive degeneration, followed by the appearance of testicular tissues after mutation of *cyp19a1a* (Nakamoto et al., 2018). In addition, the gonad phenotype of *cyp19a1a* mutant was successfully rescued to form ovary by continuous exogenous E2 treatment (Lau et al., 2016; Zhang et al., 2017; Yin et al., 2017). The ovary of rescued *cyp19a1a* mutants will restore testis after withdrawal of E2 treatment because no endogenous estrogen can be synthesized (Our unpublished data) (Fig. 3).

The majority of estrogenic activities are mediated via nuclear estrogen receptors (ER) (Klinge, 2000; Hewitt and Korach, 2002). Surprisingly, in zebrafish, knockout of single ER (*esr1*, *esr2a*, *esr2b*) displays no sex reversal. Double mutation of *esr2a/b* simultaneously led to an arrest of folliculogenesis at previtellogenic (PV, stage II) stage

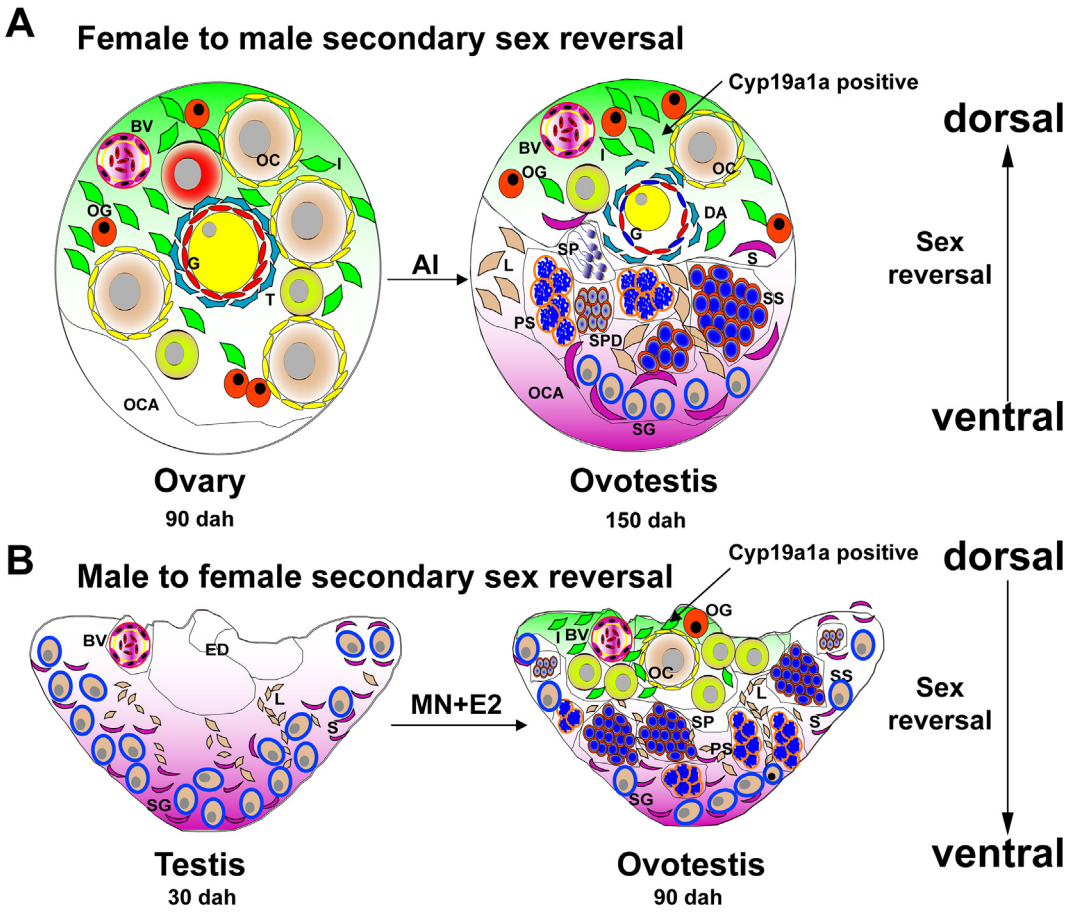

Fig. 5. Orientation of sex reversal in tilapia. A, Tilapia female ovaries were transdifferentiated into functional testes by aromatase inhibitor (AI) treatment. Female to male sex reversal was induced by AI treatment from 90 days after hatching (dah) to 150 dah in XX fish. Sex reversal happens from the ventral side (near the blood vessel) to dorsal side. B, Blockage of androgen and administration of estrogen induce transdifferentiation of testis into ovary. Differentiated XY tilapia was treated with metopirone (MN) (inhibitor of Cyp11b2, the key enzyme for 11-KT synthesis) in combination with 17β-estradiol (E2) from 30 to 90 dah. Transdifferentiation of the differentiated testis initiated from the area near the blood vessel and ended at the opposite end of the XY gonad. dah, days after hatching. PGC, primordial germ cell. BV, blood vessel. OC, oocyte. G, granulosa cells. OG, oogonia. I, interstital cells. OCA, ovary cavity. T, theca cells. ED, efferent duct. SG, spermatogonia. SS, spermatocyte. SP, sperm. PS, primary spermatocyte. L, leydig cells. S, sertoli cells. MN, metopirone.

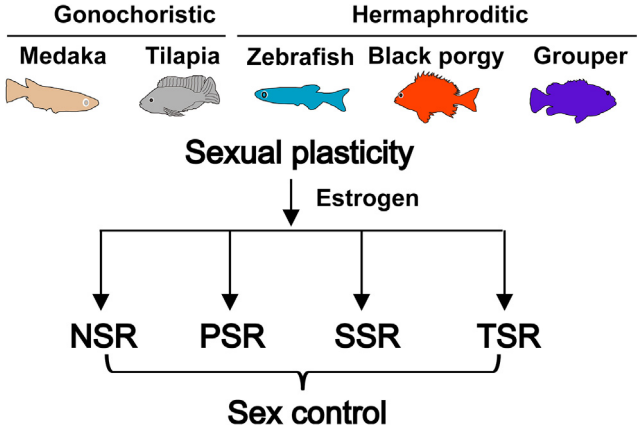

Fig. 6. Sex control in fish by modulating estrogens. Fish maintain their sexual plasticity after sex determination/differentiation in both gonochoristic and hermaphroditic fish. Endogenous estrogens play a critical role in fish sexual plasticity. Sex control in aquaculture can be achieved by modulating estrogens level through artificial primary sex reversal (PSR), secondary sex reversal (SSR), transgenic or knockout sex reversal (TSR).

followed by sex reversal from female to male (Lu et al., 2017). The authors pointed that despite all-male phenotype obtained in both *esr2a/b* and *cyp19a1a* mutants, the underlying mechanisms were obviously different. Loss of aromatase blocked ovarian differentiation whereas the lack of nERs failed to maintain female status leading to sex reversal from females to males. These genetic evidences strongly support the conclusion that Cyp19a1a is indispensable for female sex differentiation/maintenance and is a reliable early marker of ovarian differentiation in fishes.

Previous studies showed that Dmrt1 directly repressed *cyp19a1a* transcription in tilapia. Transgenic overexpression of Dmrt1 in XX tilapia resulted in decreased aromatase gene expression and reduced serum E2 level leading to sex reversal (Wang et al., 2010). In medaka, Dmy also suppresses Ad4BP/SF-1 activated *cyp19a1a* gene transcription (Wang et al., 2010). Importantly, knockdown of *dmy* resulted in sex reversal and increase of *cyp19a1a* expression (Chakraborty et al., 2016). Transforming growth factor-β(TGF-β) signal pathway is also involved in regulating aromatase/*cyp19a1a* expression. Knockdown of *amhy* in XY Patagonian pejerrey (*Odontesthes hatcheri*) resulted in up-regulation of *cyp19a1a* mRNA and the development of ovaries (Hattori et al., 2012). Knockout of *amhy* or *amhrII*, or *gsdf* in XY tilapia resulted in increased aromatase gene expression and serum E2 level, leading to sex reversal (Li et al., 2015; Jiang et al., 2016). Dysfunctional Amh signaling is responsible for up-regulation of *cyp19a1a* expression in the medaka hotei mutants (Nakamura et al., 2012).

Foxl2 is a member of the Forkhead Box (Fox) domain transcription factor. In tilapia, our studies demonstrated an important role of Foxl2 in the transcriptional regulation of *cyp19a1a*. Foxl2 up-regulates

aromatase expression *in vivo*. Mutation of *foxl2* in XX fish by TALEN resulted in sex reversal and decreased aromatase gene expression and serum E2 level (Wang et al., 2007; Li et al., 2013; Zhang et al., 2017). Heterozygous mutation of *Sf-1* decreased *cyp19a1a*, *foxl2* expression and serum E2 leading to female to male sex reversal in XX tilapia (Xie et al., 2016). In medaka, transgenic overexpression of *rspo1* up-regulate *cyp19a1a* expression and serum E2 level (Zhou et al., 2016). In clawed frog (*Xenopus laevis*), overexpression of sex determining gene *DM-W* in ZZ gonads up-regulates *Cyp19a* expression (Yoshimoto et al., 2008; Okada et al., 2009). In zebrafish, disruption of *bmp15* signaling fail to express *cyp19a1a* and thus resulted in female to male sex reversal (Dranow et al., 2016). Taken together, the male pathway genes could repress *cyp19a1a* expression and decrease estrogen level so as to promote testis formation. Conversely, the female pathway genes up-regulate *cyp19a1a* expression and increase estrogen level so as to promote ovary formation (Fig. 4).

2.5. Orientation of sex reversal

In XX tilapia, the steroidogenic (aromatase positive) cells are firstly observed in the restricted area (stromal cell) near the blood vessel (dorsal) at 5 days after hatching (dah), and the ovarian cavity is formed in ventral area, opposite to the blood vessel (dorsal) at around 35–50 dah. Oogenesis proceeds as cluster of oocytes from area adjacent to blood vessel to the ventral in ovary (Chen et al., 2016; Nakamura et al., 1989, 1998; Ruksana et al., 2011; Strüssmann and Nakamura, 2002). The differentiation of the bipotential gonad is from the dorsal side to the ventral side in XX fish.

It was found that female-to-male SSR in XX tilapia occurs from the ventral side to the dorsal side (Fig. 5A). At the early stage of female-to-male SSR, sex reversal was initiated from the germinal epithelium in the ventral side as demonstrated by the first appearance of male specific Dmrt1-positive cells (Sun et al., 2014). At the later stage, a few oocytes remained in the area near the blood vessel (dorsal area). Cyp19a1a expression was also detected in some somatic cells surrounding the remaining oocytes. Consistently, in XX *cyp19a1a* mutant medaka, ovarian tissues underwent extensive degeneration, followed by the appearance of testicular tissues on the ventral of ovaries (Nakamoto et al., 2018).

During male-to-female SSR in XY fish, the first appearance of Cyp19a1a positive cells and oocytes were observed in the area adjacent to the blood vessel (dorsal) (Fig. 5B). The oocytes were gradually increased in numbers occupying the central area with Cyp19a1a positive cells scattered as clusters of interstitial cells, and finally the oocytes appeared in the ventral area and occupied the SSR gonad. These results indicated that trans-differentiation of the differentiated testis proceeds in the same direction like ovarian differentiation, initiated from the dorsal to ventral in the XY gonad. Based on these results, we concluded that male-to-female sex reversal initiates from dorsal side (near the blood vessel) and ends at the ventral side, while female-to-male sex reversal occurs from the ventral side to dorsal side.

3. Conclusions

Fish sex exhibits striking plasticity. Down regulation of endogenous estrogen is essential for the ovarian transdifferentiation. Blockage of androgen synthesis is a critical step for induction of testicular trans-differentiation by exogenous E2, indicating that endogenous estrogen is critical for the ovarian differentiation/maintenance and androgen is critical for testicular maintenance. The male pathway genes determine testicular development by repressing *cyp19a1a* expression. Conversely, the female pathway genes determine ovarian development by up-regulating *cyp19a1a* expression. The mechanisms (target and signal pathway) for estrogens action to ovary differentiation/maintenance will be an interesting issue to be addressed in the future. Estrogen synthesis is tightly correlated with epigenetic modification of *cyp19a1a* promoter in NSR. Further studies need to investigate the relationship between epigenetic modification and PSR, SSR and TSR. Taken together, estrogens play a central role in natural sex reversal, artificially induced primary and secondary sex reversal and transgenic (knockout) sex reversal in fish. It could be widely used for sex control in aquaculture (Fig. 6).

Acknowledgments

This work was supported by Grants 31630082, 31602134 and 31772830 from the National Natural Science Foundation of China, Grant 2018YFD0901201 from the National Key Basic Research Program of China, Grant cstc2017jcyjAX0138, cstc2017shms-xdny80018 and cstc2015jcyjB80001 from the Natural Science Foundation Project of Chongqing, Chongqing Science and Technology Commission; Grant XDJK2018B026 from the Fundamental Research Funds for the Central Universities.

Competing financial interests

The authors declare no competing financial interests.

Author contributions statement

D.W, M.H, L.N. wrote the manuscript. All authors read and approved the manuscript.

References

Afonso, L.O., Wassermann, G.J., De Oliveira, R.T., 2001. Sex reversal in Nile tilapia (*Oreochromis niloticus*) using a nonsteroidal aromatase inhibitor. J. Exp. Zool. 290, 177–181.

Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol. Reprod. Dev. 67, 303–307.

Bhandari, R.K., Alam, M.A., Soyano, K., Nakamura, M., 2006. Induction of female-to-male sex change in the honeycomb grouper (*Epinephelus merra*) by 11-ketotestosterone treatments. Zool. Sci. 23, 65–69.

Chakraborty, T., Zhou, L.Y., Chaudhari, A., Iguchi, T., Nagahama, Y., 2016. Dmy initiates masculinity by altering *Gsdf/Sox9a2/Rspo1* expression in medaka (*Oryzias latipes*). Sci. Rep. 6, 19480.

Chang, C.F., Yueh, W.S., 1990. Annual cycle of gonadal histology and steroid profiles in the juvenile males and adult females of the protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture 91, 179–196.

Chang, C.F., Lee, M.F., Chen, G.R., 1994. Estradiol-17-beta associated with the sex reversal in Protandrous Black Porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. 268, 53–58.

Chang, C.F., Lau, E.L., Lin, B.Y., 1995. Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17β in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 100, 355–367.

Chen, L., Jiang, X., Feng, H., Shi, H., Sun, L., Tao, W., Xie, Q., Wang, D., 2016. Simultaneous exposure to estrogen and androgen resulted in feminization and endocrine disruption. J. Endocrinol. 228, 205–218.

Dranow, D.B., Hu, K., Bird, A.M., Lawry, S.T., Adams, M.T., Sanchez, A., Amatruda, J.F., Draper, B.W., 2016. Bmp15 Is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in Zebrafish. PLoS Genet. 12, e1006323.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364.

Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat. Toxicol. 67, 105–126.

Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., Mcmeel, O.M., Martin, S.A., Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev. 54, 154–162.

Guiguen, Y., Jalabert, B., Thouard, E., Fostier, A., 1993. Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass, *Lates calcarifer*. Gen. Comp. Endocrinol. 92, 327–338.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165, 352–366.

Hattori, R.S., Murai, Y., Oura, M., Masuda, S., Majhi, S.K., Sakamoto, T., Fernandino, J.I., Somoza, G.M., Yokota, M., Strüssmann, C.A., 2012. A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. Proc. Natl. Acad. Sci. U.S.A. 109, 2955–2959.

Hewitt, S.C., Korach, K.S., 2002. Estrogen receptors: structure, mechanisms and function. Rev. Endocr. Metab. Disord. 3, 193–200.

Huang, W., Zhou, L., Li, Z., Gui, J.F., 2009. Expression pattern, cellular localization and

promoter activity analysis of ovarian aromatase (Cyp19a1a) in protogynous hermaphrodite red-spotted grouper. Mol. Cell Endocrinol. 307, 224–236.
Huang, M., Wang, Q., Chen, J., Chen, H., Xiao, L., Zhao, M., Zhang, H., Li, S., Liu, Y., Zhang, Y., Lin, H., 2018. The co-administration of estradiol/17α-methyltestosterone leads to male fate in the protogynous orange-spotted grouper, *Epinephelus coioides*. Biol. Reprod. https://doi.org/10.1093/biolre/ioy211.
Iwamatsu, T., Kobayashi, H., Sagegami, R., Shuo, T., 2006. Testosterone content of developing eggs and sex reversal in the medaka (*Oryzias latipes)*. Gen. Comp. Endocrinol. 145, 67–74.
Jiang, D.N., Yang, H.H., Li, M.H., Shi, H.J., Zhang, X.B., Wang, D.S., 2016. *gsdf* is a downstream gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia. Mol. Reprod. Dev. 83, 497–508.
Komatsu, T., Nakamura, S., Nakamura, M., 2006. Masculinization of female golden rabbitfish *Siganus guttatus* using an aromatase inhibitor treatment during sex differentiation. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 143, 402–409.
Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S.I., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J. Mol. Endocrinol. 23, 167–176.
Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. Mol. Reprod. Dev. 59, 359–370.
Klinge, C.M., 2000. Estrogen receptor interaction with co-activators and co-repressors. Steroids 65, 227–251.
Lau, E.S., Zhang, Z., Qin, M., Ge, W., 2016. Knockout of Zebrafish ovarian aromatase gene (cyp19a1a) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. Sci. Rep. 6, 37357.
Li, M.H., Yang, H.H., Li, M.R., Sun, Y.L., Jiang, X.L., Xie, Q.P., Wang, T.R., Shi, H.J., Sun, L.N., Zhou, L.Y., Wang, D.S., 2013. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. Endocrinology 154, 4814–4825.
Li, M., Yang, H., Zhao, J., Fang, L., Shi, H., Li, M., Sun, Y., Zhang, X., Jiang, D., Wang, D., 2014. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics 197, 591–599.
Li, M., Sun, Y., Zhao, J., Shi, H., Zeng, S., Ye, K., Jiang, D., Zhou, L., Sun, L., Tao, W., Nagahama, Y., Kocher, T.D., Wang, D., 2015. A tandem duplicate of anti-müllerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile Tilapia, *Oreochromis niloticus*. PLoS Genet. 11, e1005678.
Li, M.H., Wang, D.S., 2017. Gene editing nuclease and its application in tilapia. Sci. Bull. 62, 165–173.
Lu, H., Cui, Y., Jiang, L., Ge, W., 2017. Functional analysis of nuclear estrogen receptors in Zebrafish reproduction by genome editing approach. Endocrinology 158, 2292–2308.
Manolakou, P., Lavranos, G., Angelopoulou, R., 2006. Molecular patterns of sex determination in the animal kingdom: a comparative study of the biology of reproduction. Reprod. Biol. Endocrinol. 4, 59.
McAllister, B.G., Kime, D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65, 309–316.
Mei, J., Gui, J.F., 2015. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. Sci. China Life Sci. 58, 124–136.
Murata, R., Kobayashi, Y., Karimata, H., Kishimoto, K., Kimura, M., Nakamura, M., 2014. Transient sex change in the immature Malabar grouper, *Epinephelus malabaricus*, androgen treatment. Biol. Reprod. 9, 25.
Nagahama, Y., 2002. Ontogeny and plasticity of sex determination/gonadal differentiation in fishes. Biol. Reprod. 66, 75–76.
Nagahama, Y., 2000. Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish. In: Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., Stefansson, S.O. (Eds.), Proceedings of the 6th International Symposium on Reproductive Physiology of Fish. Institute of Marine Research and University of Bergen, Bergen, Norway, pp. 211–222.
Nakamura, M., Hourigan, T.F., Yamauchi, K., Nagahama, Y., Grau, E., 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. Environ. Biol. Fish. 24, 117–136.
Nakamura, S., Watakabe, I., Nishimura, T., Picard, J.Y., Toyoda, A., Taniguchi, Y., di Clemente, N., Tanaka, M., 2012. Hyperproliferation of mitotically active germ cells due to defective anti-Müllerian hormone signaling mediates sex reversal in medaka. Development 139, 2283–2287.
Nakamoto, M., Shibata, Y., Ohno, K., Usami, T., Kamei, Y., Taniguchi, Y., Todo, T., Sakamoto, T., Young, G., Swanson, P., Naruse, K., Nagahama, Y., 2018. Ovarian aromatase loss-of-function mutant medaka undergo ovary degeneration and partial female-to-male sex reversal after puberty. Mol. Cell Endocrinol. 460, 104–122.
Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genet. 7, e1002447.
Navarro-Martín, L., Blázquez, M., Piferrer, F., 2009. Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. Gen. Comp. Endocrinol. 160, 3–11.
Nakamura, M., Kobayashi, T., Chang, X.T., Nagahama, Y., 1998. Gonadal sex differentiation in teleost fish. J. Exp. Zool. 281, 362–372.
Okada, E., Yoshimoto, S., Ikeda, N., Kanda, H., Tamura, K., Shiba, T., Takamatsu, N., Ito, M., 2009. Xenopus W-linked DM-W induces Foxl2 and Cyp19 expression during ovary formation. Sex Dev. 23, 38–42.
Paul-Prasanth, B., Bhandari, R.K., Kobayashi, T., Horiguchi, R., Kobayashi, Y., Nakamoto, M., Shibata, Y., Sakai, F., Nakamura, M., Nagahama, Y., 2013. Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists. Sci. Rep. 3, 2862.
Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994. Brief treatment with an aromatase inhibitor during sex-differentiation causes chromosomally female salmon to develop as normal, functional males. J. Exp. Zool. 270, 255–262.
Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.
Ruksana, S., Alam, M.A., Kobayashi, Y., Nakamura, M., 2011. Differentiation of steroid-producing cells and folliculogenesis in the developing ovary of the Nile tilapia *Oreochromis niloticus*. Zool. Sci. 28, 845–852.
Shao, C., Li, Q., Chen, S., Zhang, P., Lian, J., Hu, Q., Sun, B., Jin, L., Liu, S., Wang, Z., Zhao, H., Jin, Z., Liang, Z., Li, Y., Zheng, Q., Zhang, Y., Wang, J., Zhang, G., 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res. 24, 604–615.
Shi, H., Gao, T., Liu, Z., Sun, L., Jiang, X., Chen, L., Wang, D., 2017. Blockage of androgen and administration of estrogen induce transdifferentiation of testis into ovary. J. Endocrinol. 233, 65–80.
Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelson, C.R., Bulun, S.E., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355.
Strüssmann, C.A., Nakamura, M., 2002. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol. Biochem. 26, 13–29.
Sun, L.N., Jiang, X.L., Xie, Q.P., Yuan, J., Huang, B.F., Tao, W.J., Zhou, L.Y., Nagahama, Y., Wang, D.S., 2014. Transdifferentiation of differentiated ovary into functional testis by long term treatment of aromatase inhibitor in Nile tilapia. Endocrinology 155, 1476–1488.
Takatsu, K., Miyaoku, K., Roy, S.R., Murono, Y., Sago, T., Itagaki, H., Nakamura, M., Tokumoto, T., 2013. Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. Sci. Rep. 3, 3400.
Tao, W., Yuan, J., Zhou, L., Sun, L., Sun, Y., Yang, S., Li, M., Zeng, S., Huang, B., Wang, D., 2013. Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes. PLoS One. 8, e63604.
Tsai, Y.J., Lee, M.F., Chen, C.Y., Chang, C.F., 2011. Development of gonadal tissue and aromatase function in the protogynous orange-spotted grouper *Epinephelus coioides*. Zool. Stud. 50, 693–704.
Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2004. An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 137, 11–20.
Vizziano, D., Baron, D., Randuineau, G., Mahè, S., Cauty, C., Guiguen, Y., 2008. Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. Biol. Reprod. 78, 939–946.
Wang, D.S., Kobayashi, T., Zhou, L.Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol. Endocrinol. 21, 712–725.
Wang, D.S., Zhou, L.Y., Kobayashi, T., Matsuda, M., Shibata, Y., Sakai, F., Nagahama, Y., 2010. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. Endocrinology 151, 1331–1340.
Wang, Q., Liu, Y., Peng, C., Wang, X., Xiao, L., Wang, D., Chen, J., Zhang, H., Zhao, H., Li, S., Zhang, Y., Lin, H., 2017. Molecular regulation of sex change induced by methyltestosterone -feeding and methyltestosterone -feeding withdrawal in the protogynous orange-spotted grouper. Biol. Reprod. 97, 324–333.
Wang, Q., Huang, M., Peng, C., Wang, X., Xiao, L., Wang, D., Chen, J., Zhao, H., Zhang, H., Li, S., Yang, H., Liu, Y., Lin, H., Zhang, Y., 2018. MT-feeding-induced impermanent sex reversal in the orange-spotted grouper during sex differentiation. Int. J. Mol. Sci. 19 (9).
Wu, G.C., Tomy, S., Nakamura, M., Chang, C.F., 2008. Dual roles of cyp19a1a in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod. 79, 1111–1120.
Wu, G.C., Tey, W.G., Li, H.W., Chang, C.F., 2015. Sexual fate reprogramming in the steroid-induced bi-directional sex change in the protogynous orange-spotted grouper, *Epinephelus coioides*. PLoS One 10, e0145438.
Wu, G.C., Li, H.W., Huang, C.H., Lin, H.J., Lin, C.J., Chang, C.F., 2016. The testis is a primary factor that contributes to epigenetic modifications in the ovaries of the protandrous black porgy, *Acanthopagrus schlegelii*. Biol. Reprod. 94, 132.
Wu, G.C., Chang, C.F., 2017. Primary males guide the femaleness through the regulation of testicular Dmrt1 and ovarian Cyp19a1a in protandrous black porgy. Gen. Comp. Endocrinol S0016-6480(16)30385-9.
Xie, Q.P., He, X., Sui, Y.N., Chen, L.L., Sun, L.N., Wang, D.S., 2016. Haploinsufficiency of SF-1 causes female to male sex reversal in Nile Tilapia, *Oreochromis niloticus*. Endocrinology 157, 2500–2514.
Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., Shiba, T., Ito, M., 2008. A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. Proc. Natl. Acad. Sci. U.S.A. 105, 2469–2474.
Yin, Y., Tang, H., Liu, Y., Chen, Y., Li, G., Liu, X., Lin, H., 2017. Targeted disruption of aromatase reveals dual functions of cyp19a1a during sex differentiation in zebrafish. Endocrinology 158, 3030–3041.

Zhang, W., Zhang, Y., Zhang, L., Zhao, H., Li, X., Huang, H., Lin, H., 2007. The mRNA expression of P450 aromatase, gonadotropin beta-subunits and FTZ-F1 in the orange-spotted grouper (*Epinephelus coioides*) during 17alpha-methyltestosterone-induced precocious sex change. Mol. Reprod. Dev. 74, 665–673.

Zhang, Y., Zhang, S., Lu, H., Zhang, L., Zhang, W., 2014. Genes encoding aromatases in teleosts: evolution and expression regulation. Gen. Comp. Endocrinol. 205, 151–158.

Zhang, Y., Zhang, S., Liu, Z., Zhang, L., Zhang, W., 2013. Epigenetic modifications during sex change repress gonadotropin stimulation of cyp19a1a in a teleost rice field eel (*Monopterus albus*). Endocrinology 154, 2881–2890.

Zhang, X., Li, M., Ma, H., Liu, X., Shi, H., Li, M., Wang, D., 2017. Mutation of foxl2 or cyp19a1a results in female to male sex reversal in XX Nile Tilapia. Endocrinology 158, 2634–2647.

Zhou, L., Charkraborty, T., Zhou, Q., Mohapatra, S., Nagahama, Y., Zhang, Y., 2016. Rspo1-activated signalling molecules are sufficient to induce ovarian differentiation in XY medaka (*Oryzias latipes*). Sci. Rep. 6, 19543.