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The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegeli*

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Abstract Hermaphrodites have both sexes during their life, including an initial primary sex determination and in later stage maintenance one of the sexual fates (secondary sex determination). Sex change (secondary sex determination) occurs in animals, but it is lost in amphibians through, mammals in vertebrates. Teleosts have various strategies and mechanisms of sex determination including genetic and environmental cues. However, the mechanisms by which the cues guide sex change are complicated in fish. This manuscript reviews our understanding of these processes in protandrous black porgy at the gonadal and neuroendocrine levels. Our studies addressed the process of sex change through brain-pituitary-gonad axis, and then secondary sex determination was switched by the fate of testis.

Keywords Estrogen · Hermaphroditism · Sex change · Sex differentiation

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Introduction

Sex determination is an ancient and universal feature in animals. According to the type of sex organ in organisms, it can be briefly classified into two groups: gonochorism (which has fixed sexes) and hermaphroditism (which can have both sexes during their life). Functional hermaphroditism occurs in animals as diverse as annelids, echinoderms, crustaceans, mollusks, and fishes (Polikansky 1982). However, it is lost from amphibians through, mammals in vertebrates. Furthermore, fishes with many species and have different kinds of strategies and mechanisms. Thus, fish provide a unique model for studying the mechanism of hermaphroditic sex change in vertebrates. In gonochorism, sex is determining at early developmental stage. In mammals, the formation of the testis and subsequent male development are initiated by the sex-determining *SRY* gene on the Y chromosome (Swain and Lovell-Badge 1999). By contrast, the *doublesex and mab-3 related transcription factor 1* (*DMRT1*) is a gonad-specific transcription factor related to the invertebrate sexual regulator. *DMRT1* (Swain and Lovell-Badge 1999) and its paralogues are candidates for sex-determining genes in non-mammalian vertebrates, including the chicken Z-linked *DMRT1* (Smith et al. 2009), the frog W-linked *DM-W* (Yoshimoto et al. 2008), and the medaka fish *Y-linked dmy/dmrt1by* (Matsuda et al. 2002; Nanda et al. 2002). Furthermore, *DMRT1* has been suggested to be an important transcriptional regulator of male

differentiation that is required for testicular development in vertebrates (Herpin and Schartl 2011). Thus, *dmrt1* function might be conserved in the regulation of male development not only in gonochorism but also in hermaphroditism.

In fishes, natural sex change implies the phenomenon of a sequential expression of male and female phenotypic sexes in the normal gonadal ontogeny of hermaphroditism. According to the sexual phase of ovotestes, sequential hermaphroditism can be classified into two groups: protandrous (male-to-female sex change), protogynous (female-to-male sex change). Therefore, sex is determined in initial gonadal differentiation (primary sex determination) and in later sexual phase maintenance (secondary sex determination) in hermaphroditic fish. Black porgies are functional males for the first 2 years of life, but about 50% of them transform into females during the third year. Therefore, black porgies are a unique model to study sex differentiation of the gonad and development of bisexual gonad especially due to the interaction between testicular and ovarian tissues in the gonad.

Maleness and femaleness

Ovotestis is observed in 3- to 4-month-old fish (Fig. 1a). The first phase of ovotestis is maleness (Fig. 1a). The development of testicular tissue begins after the gonad differentiation in 0^+-year-old (<1-year-old) fish, starting with the proliferation of spermatogonia and spermatogenesis, and then fish become mature in first reproductive season (1-year-old fish) and second reproductive season as shown in Fig. 1a (Lee et al. 2008; Wu et al. 2008b). Male-related genes (*dmrt1*, *amh*, *amhr2*, *and sf1*) were significantly increased after testicular differentiation and maintenance at high expression level during maleness period as shown in Fig. 1b (Wu et al. 2008a, b, 2010, 2012). The expressions of male-related genes (Fig. 1b) were significantly decreased before male-to-female sex change (Wu et al. 2008a, b, 2010, 2012). These fish will enter full femaleness and subsequently change sex by developing vitellogenic oocytes when approaching the third reproductive season in 3-year-old fish (Fig. 1a; Wu et al. 2005). This suggests the existence of a switch between the testis and ovary in response to the external stimulus

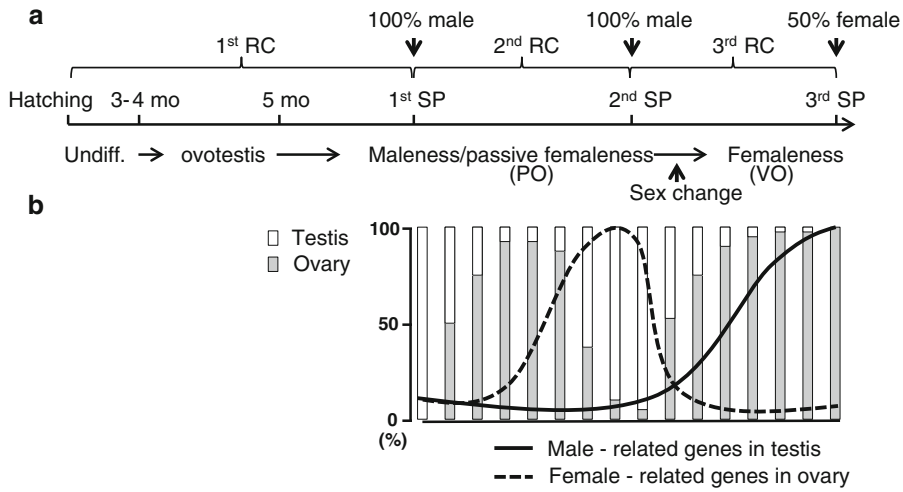

Fig. 1 Gene profiles in the testicular and ovarian tissues during natural sex change from 0^+- to 3-year-old fish. **a** Annual profile of sexual fate from 0^+- to 3-year-old fish. Ovotestis became the maleness at the first reproductive cycle (RC), and fish were maintained at the maleness with the passive femaleness (oocytes remained at the primary oocyte) at the second reproductive cycle. Fish (50%) were entered femaleness (sex change was occurred and oocytes were reached to vitellogenesis) at the third reproductive cycle. **b** The profile of gonadal tissue (the relative % area between testis and ovary in the ovotestis; testis in *white* and ovary in *gray*) from the second reproductive cycle (*RC*) to third reproductive cycle fish in relation to the development of bisexual gonad. Relative profile of gene transcripts in testis (male-related genes) and ovary (female-related genes) with a differential expression at different sexual fates (maleness and femaleness). *Undiff* undifferentiation, *PO* primary oocyte, *VO* vitellogenic oocyte, *SP* spawning season, *RC* reproductive cycle

so that only one type of gonad will dominantly become active, and the other will recede to a rudimentary stage. The expression of gonadal *amh* is higher in males than in females during sex differentiation in flounder (Yoshinaga et al. 2004), zebrafish (Rodríguez-Marí et al. 2005), and tilapia (Ijiri et al. 2008). Conversely, in medaka fish, *amh* and *amhr2* have no sexually dimorphic expression during gonadal differentiation (Klüver et al. 2007), and the loss-of-function of *amh* and *amhr2* resulted in the suppression of germ cell proliferation during gonadal differentiation in both sexes (Shiraishi et al. 2008). Moreover, in medaka fish, over 50% of genetic males undergo sex reversal that have the *amhr2*-deficiency (Morinaga et al. 2007). These data indicate that the function of *amh* and *amhr2* is important to maintain germ cell proliferation by mitosis in male gonad. Other male gene such as *sox9* is also important for gonadal development. However, several fish species have isoforms of *sox9*, including *sox9a* and *sox9b* in zebrafish (Rodríguez-Marí et al. 2005) and catfish (Raghuveer and Senthilkumaran 2010), or *sox9a* and *sox9a2* in medaka (Nakamoto et al. 2005). According to the differential expression pattern of *sox9a* and *sox9b* in gonads, indicating that *sox9a* retained its function in testicular development, *sox9b* might have a new role to play in ovary.

Estrogen and femaleness

Increased plasma estradiol (E_2) levels have been correlated with the natural sex change in the third reproductive cycle (Wu et al. 2005). In addition, the natural sex change can be blocked by long-term aromatase inhibitor (AI) administration (Lee et al. 2002). However, vitellogenic oocytes were infrequently observed following long-term treatment with E_2 in 0^+- and 1^+-year-old fish (Chang et al. 1995a; Lee et al. 2004; Wu et al. 2008a, b). In addition, E_2-induced male-to-female sex change is a reversible process in 0^+- and 1^+-year-old fish (Chang and Lin 1997; Lee et al. 2004; Wu et al. 2008a; Wu and Chang 2009). Testicular tissue regenerated and ovarian tissue degenerated after E_2-withdrawal in E_2-induced sex change. These results provide an insight that E_2-induced ovarian development might be a temporal femaleness. At molecular level, our data also strongly confirmed this hypothesis. Steroidogenesis-related factors (*sf-1*, *star*, *cyp11a1*, *hsd3b1*, and *cyp19a1a* in Fig. 1b) were significantly increased in ovarian tissue during natural sex change in 2^+- to 3-year-old fish (Wu et al. 2008a). However, the genes expressed at lower levels were detected in the ovarian tissue of E2-induced black porgy as compared to the natural sex-changed fish (Wu et al. 2008a). The lower expression of steroidogenesis-suppressing factor (*dax-1*) was observed in natural sex-changed ovary and higher expression in E_2-induced ovary (Wu et al. 2008a). On the other hand, E_2- and aromatase inhibitor (AI)-treated fish had a well-developed ovarian tissue in 0^+-year-old fish (Wu et al. 2008b). On the basis of Pcna staining results, the first step of ovarian development (with high oogonia proliferating activity) is starting after the silence of testicular development (without spermatogonia proliferating activity) in E_2- and AI-treated groups in 0^+-year-old fish (Wu et al. 2008b). Thus, estrogen might be important for early testicular development as well as ovarian development.

Passive femaleness

On the basis of proliferation and apoptosis activities, the first sign of the shift from maleness to femaleness was the high proliferating activity (increased number of Pcna-positive spermatogonia) in testicular tissue, and then the high apoptosis (increased number of TUNEL-positive oocytes) in ovarian tissue (Wu et al. 2010). This result provides an idea that femaleness might be controlled by the testicular tissue. We further used surgery to remove testicular part of ovotestis in 1^+-year-old fish. The testis-removed fish entered full femaleness phase (with vitellogenic and mature oocytes) in the second reproductive cycle in 2-year-old fish (Wu et al. 2008a; Wu and Chang 2009). In addition, cellular (significant increase in oogonia proliferating activity) and molecular events (high expression of early developing gene in ovary, *wnt4)* in testis-removed fish revealed that the testis could be a dominant factor of the switch of femaleness (Wu and Chang 2009). On the other hand, the inhibition on early testis development by exogenous E_2-/AI-administration could induce ovarian development in 0^+-year-old fish (Wu et al. 2008b). A high dose of E_2 induced ovarian development (due to the inhibition of testicular tissue by high E_2 treatment), while a low dose promoted testis growth (Chang et al. 1995a, b; Wu

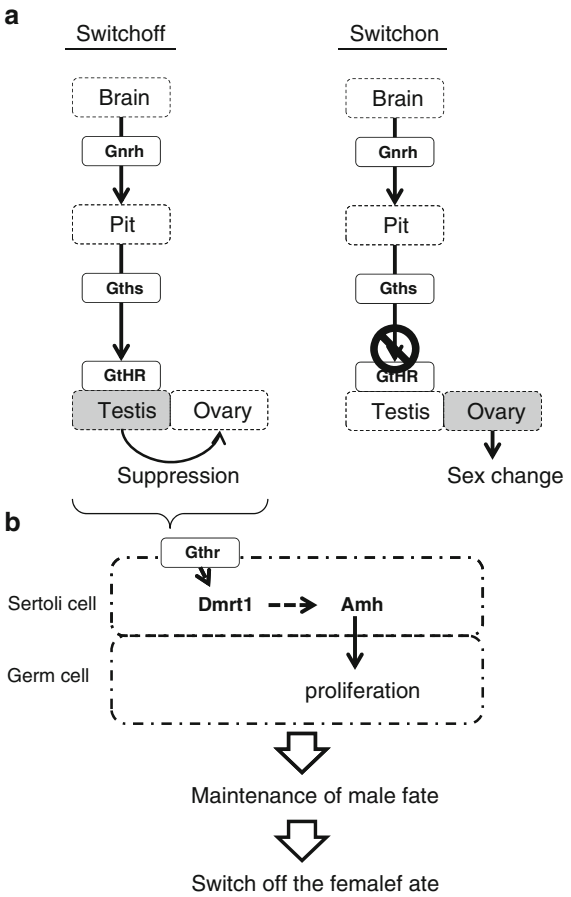

Fig. 2 The sexual fates are controlled by the brain-pituitary-gonad axis. **a** The testicular development is regulated by Gnrh-gonadotropins (Gths)-testis axis. In further, the sex switch is controlled by male fate that testis is a dominant ovary-suppressing factor. The fish will maintain at the male fate, and the sex change will be switched off if the testis receives the Gths (such as luteinizing hormone) signal. In contrast, sex change is switched on when the testis is entering the regressive stage. **b** Our studies established that Sertoli cell Dmrt1 is a key regulator for testis development and regulates spermatogonia proliferation through Amh during testis development. In addition, Dmrt1 is important for later testis growth/development (related to the inhibition of ovarian development and sex change) by controlling sexual fate. *GtHR* gonadotropin receptor, *Pit* pituitary, *Amh* anti-müllerian hormone

et al. 2008b). Thus, the development of femaleness might be a passive process in black porgy (Fig. 2a).

Femaleness follows by the spontaneous regression of testis

According to the histological results, the significant difference between suppressed testis (the gonad prior to sex change during the post- and non-spawning season in 1^+- and 2^+-year-old fish) and regressed testis (the gonad is sexually changed to an ovary) is the number of germ cells. The germ cell number is significantly decreased in the testis after sex change compared with the suppressed testis (Lee et al. 2008). However, the germ cell loss induced by busulfan treatment did not reverse the sexual fate (from maleness to femaleness) in 0^+-year-old fish (Wu et al. 2012). Male-related marker genes such as *amh* and *dmrt1* were consistently expressed in the busulfan-treated testis (Wu et al. 2012). In catfish, MT-induced ovotestis has a high *dmrt1* expression in surrounding cells (Sertoli cell) of germ cells (Raghuveer and Senthilkumaran 2009). These results suggest that testicular fate might be controlled by Sertoli cells. Our data also showed that *dmrt1* was consistently expressed in Sertoli cells and differentially and more expressed in the testis of the future male (destined to a male 6 months later) as compared to the future female fish (destined to a female 6 months later) at 2^+-year age (He et al. 2003; Wu et al. 2012). We further used shRNA to knockdown the *dmrt1* expression in 0^+-year-old fish. The knockdown fish had a regressive testis with the loss of germ cells (the germ cell marker, Vasa, cannot be detected in testis), and ovary was well developed in 0^+-year-old fish (Wu et al. 2012). Thus, the femaleness is switched on by the regression of testicular development through the diminished *dmrt1* expression (Fig. 2b).

The role of brain-pituitary-gonad axis in the maintenance of maleness

Further results revealed that the testis development was a key regulator for male-to-female sex change in the ovotestis (Wu et al. 2009). Nevertheless, it will be important to ask what signals induce the testis development of maleness. High levels of serum luteinizing hormone (Lh) were appeared during the natural sex change in 2^+- to 3-year-old black porgy (Du et al. 2005; Lee et al. 2004). Increased serum Lh levels were also detected during the natural sex change from an "E_2-induced female" to a male in 1-year-old black porgy (Lee et al. 2004). In addition, the expression of gonadotropin receptor is higher in testicular tissue than in ovarian tissue (Du et al. 2005). hCG (human chorionic gonadotropin) could have a different response of ovotestis in different

stages, including steroidogenesis-related enzymes, *amh* and *amhr2* (Wu et al. 2010). We suggest that the testicular/ovarian tissue in a single bisexual gonad has differential responses to the endogenous stimulation. Furthermore, in vivo LHRH (luteinizing hormone-releasing hormone) and in vitro hCG could up-regulate *dmrt1* expression in testicular tissue of black porgy (Wu et al. 2012). In vivo androgen also stimulated *dmrt1* expression in catfish testis (Raghuveer et al. 2005). These results confirm our hypothesis that the development of femaleness is a passive process that is controlled by the testis through the brain (Gnrh)-pituitary (gonadotropins)-gonad (Dmrt1) axis in black porgy (Fig. 2).

Conclusion

Protandrous black porgy with predictably and dramatically anatomical changes in the reproductive system makes a unique model fish to study sex differentiation and development. This review described the sequential expressions of certain male and female-related genes. According to the data from sex change in the testis-removed fish, we suggest that maintaining the male phase will inhibit sex change in the bisexual gonad. This male phase maintenance may be regulated by the brain (Gnrh)-pituitary (gonadotropins)-gonad (Dmrt1) axis (Fig. 2).

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