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Estrogen alters *gonadal soma-derived factor* (*Gsdf*)/*Foxl2* expression levels in the testes associated with testis-ova differentiation in adult medaka, *Oryzias latipes*

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ABSTRACT

Testis-ova differentiation in sexually mature male medaka (*Oryzias latipes*) is easily induced by estrogenic chemicals, indicating that spermatogonia persist in sexual bipotentiality, even in mature testes in medaka. By contrast, the effects of estrogen on testicular somatic cells associated with testis-ova differentiation in medaka remain unclear. In this study, we focused on the dynamics of sex-related genes (*Gsdf, Dmrt1*, and *Foxl2*) expressed in Sertoli cells in the mature testes of adult medaka during estrogen-induced testis-ova differentiation. When mature male medaka were exposed to estradiol benzoate (EB; 800 ng/L), testis-ova first appeared after EB treatment for 14 days (observed as the first oocytes of the leptotene-zygotene stage). However, the testis remained structurally unchanged, even after EB treatment for 28 days. Although *Foxl2* is a female-specific sex gene, EB treatment for 7 days induced *Foxl2*/FOXL2 expression in all Sertoli cell-enclosed spermatogonia before testis-ova first appeared; however, *Foxl2* was not detected in somatic cells in control testes. Conversely, Sertoli-cell-specific *Gsdf* mRNA expression levels significantly decreased after EB treatment for 14 days, and no changes were observed in DMRT1 localization following EB treatment, whereas *Dmrt1* mRNA levels increased significantly. Furthermore, after EB exposure, FOXl2 and DMRT1 were co-localized in Sertoli cells during testis-ova differentiation, although FOXL2 localization was undetectable in Sertoli-cell-enclosed apoptotic testis-ova, whereas DMRT1 remained localized in Sertoli cells. These results indicated for the first time that based on the expression of female-specific sex genes, feminization of Sertoli cells precedes testis-ova differentiation induced by estrogen in mature testes in medaka; however, complete feminization of Sertoli cells was not induced in this study. Additionally, it is suggested strongly that *Foxl2* and *Gsdf* expression constitute potential molecular markers for evaluating the effects of estrogenic chemicals on testicular somatic cells associated with estrogen-induced testis-ova differentiation in mature male medaka.

1. Introduction

The widespread impact of environmental pollution on animal life has increased global concern and awareness regarding the adverse effects of synthetic hormones in the environment. Endocrine disruptors and synthetic hormones, such as estrogen and estrogenic chemicals, can

Abbreviations: Gsdf, gonadal soma-derived factor; *Dmy*, DM domain gene on the Y chromosome; *Dmrt1*, doublesex/mab-3 related transcription factor-1; *Amh*, anti-müllerian hormone; qPCR, quantitative PCR; EB, estradiol benzoate; EDC, endocrine disrupting chemicals; PBS, phosphate-buffered saline

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significantly impair sexual development and reproductive function in diverse animal populations, particularly in aquatic organisms, which are easily exposed to estrogenic chemicals in polluted water. The effects of estrogen and estrogenic chemicals on altering sexual characteristics and reproduction have been previously studied in several fish populations (Sumpter and Jobling, 1995; Lange et al., 2009).

In some lower vertebrates, oocyte-like cells occur spontaneously in the mature testis, resulting in the formation of an intersex condition called testis-ova (Atz, 1964; Kobayashi et al., 2015). Several studies, including laboratory experiments, have reported the occurrence of testis-ova in various fish species, suggesting that testis-ova are induced by exposure to estrogenic chemicals (Egami, 1955a; Urushitani et al., 2007; Lange et al., 2009). Intersex has been suggested as an important determinant of reproductive success in fish living in effluent-contaminated rivers (Harris et al., 2011). The occurrence of testis-ova was described in wild-type roach, *Rutilus rutilus*, living downstream of sewage-treatment discharges. Additionally, the exposure of roach to sewage effluent causes altered sexual development, resulting in reduced fertility (Jobling et al., 1998; Lange et al., 2011). In wild roach and laboratory medaka (*Oryzias latipes*), the occurrence of testis-ova is accompanied by the disruption of spermatogenesis, suggesting diminished fertility (Egami, 1955a; Jobling et al., 2006).

The occurrence of testis-ova is one of the endpoints used to assess the effects of endocrine-disrupting chemicals (Hirakawa et al., 2012). To elucidate the disruptive effects of estrogenic chemicals, testis-ova development was examined by histological analysis, although the detailed mechanisms of estrogenic chemical-induced testis-ova differentiation remain unclear. In the teleost fish, medaka, testis-ova can be easily induced by various methods, including exposure to estrogenic compounds (Egami, 1955a,b,c,d,e); however, spontaneous differentiation of testis-ova in this species under laboratory conditions has not been reported. Testis-ova development in medaka can be detected using oocyte-specific marker genes, including *42Sp50* and *Zpc5*, specifically expressed in correlation with the presence of testis-ova (Kinoshita et al., 2008; Hirakawa et al., 2012). However, gene expression profiles of testicular somatic cells associated with testis-ova differentiation remain obscure.

Medaka has an XX/XY sex determination system, with a male sex-determining gene, *Dmy* (also known as *Dmrt1(Y)b*) (Matsuda et al., 2002; Nanda et al., 2002). Recent studies suggested that the gene encoding gonadal soma-derived factor (*Gsdf*) is a downstream target gene closely related to *Dmy* and its product a member of the transforming growth-factor beta (TGF-β) superfamily based on its expression profile and potential for the induction of testis differentiation by a gain-of-function mutation (Shibata et al., 2010; Myosho et al., 2012; Imai et al., 2015; Zhang et al., 2016; Horie et al., 2016). In medaka (*O. latipes*), initial morphological sex differentiation is defined by the difference in germ-cell number between both sexes during hatching (Kobayashi et al., 2004). Additionally, *Gsdf* disruption causes ovarian differentiation in an inbred XY Hd-rR strain of medaka at an early developmental stage (at hatching) (Imai et al., 2015). Similar to other testis-differentiation factors, doublesex/mab-3-related transcription factor-1 (encoded by *Dmrt1*) was identified in Hd-rR fish based on an analysis of a loss-of-function mutant wherein XY gonads differentiate into testes up to 5-days post-hatching, after which they transdifferentiate into ovaries (Masuyama et al., 2012). Together, these facts suggest that *Gsdf* is critical in directing bipotential gonads in early sex-differentiation stages of *O. latipes*, whereas *Dmrt1* functions during later stages of testis differentiation and development (Imai et al., 2015; Zhang et al., 2016). In sex-reversed medaka (induced by exposure to steroidogenic chemicals), *Gsdf* and *Dmrt1* expression levels are downregulated and upregulated in XY and XX sex reversals, respectively (Kobayashi et al., 2004; Shibata et al., 2010; Horie et al., 2016), although it remains unclear whether *Gsdf* and *Dmrt1* expression levels are affected in correlation with estrogen-induced testis-ova differentiation.

In teleost fish, including medaka, spermatogenesis progresses clonally within a cyst consisting of Sertoli cells surrounding germ cells. Testis-ova are also differentiated from spermatogonia within a cyst (Egami, 1955a,b,c,d,e; Shibata and Hamaguchi, 1988; Kobayashi et al., 2004). In medaka, *Gsdf* and *Dmrt1* were detected in a Sertoli cell lineage specifically (Kobayashi et al., 2004; Horie et al., 2016). However, *Foxl2* mutation and knockout studies elucidated that *Foxl2* plays a critical role in ovarian differentiation (Ottolenghi et al., 2005; Cocquet et al., 2003; Schmidt et al., 2004; Uda et al., 2004), and *Foxl2* is also expressed in germ-cell-supporting cells in ovaries (but not in testis) in mammals and fish, including medaka (Govoroun et al., 2004; Hudson et al., 2005; Nakamoto et al., 2006; Wang et al., 2007). These reports suggested that in germ-cell-supporting cells, *Gsdf* and *Dmrt1* expression reveals male characteristics, whereas additional expression of *Foxl2* reveals female characteristics. Therefore, to understand the effects of estrogen on testicular somatic cells associated with testis-ova differentiation, we specifically focused on the expression dynamics of the sex-related genes *Gsdf/Dmrt1/Foxl2* in Sertoli cells as germ cell-supporting cells during estrogen-induced testis-ova differentiation in mature male medaka. Based on the sex-related gene expression, our study indicated for the first time that feminization of Sertoli cells precedes testis-ova differentiation in mature medaka testes.

2. Materials and methods

2.1. Fish

The inbred Hd-rR strain of medaka (*O. latipes*) was supplied by the National BioResource Project Medaka (NBRP Medaka; Okazaki, Japan), under the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan (https://www.shigen.nig.ac.jp/medaka/). Medaka was maintained in the aquaria at University of Shizuoka (Shizuoka, Japan) under an artificial photoperiod of 14-h/10-h light/dark at 25 ± 1 °C.

To specifically detect DMRT1 localization in Sertoli cells before and after estradiol benzoate (EB) exposure, we analyze localization in XX sex-reversed males. In our previous study, *Dmrt1* co-localized with *Dmy* in Sertoli-cell-lineage cells (Kobayashi et al., 2004). Because the sequence recognized by the antibody is similar in both DMY and DMRT1 (Kobayashi et al., 2004; Horie et al., 2016), it is possible that the DMRT1 antibody can detect both DMRT1and DMY in XY testes. To clarify whether DMRT1 and FOXL2 co-localization occurs in testicular cells during testis-ova differentiation after EB exposure, we analyzed XX sex-reversed males. To obtain XX sex-reversed males in an Hd-rR inbred strain, fertilized eggs were incubated in dechlorinated water at 32 ± 1 °C from fertilization to hatching and maintained at 25 ± 1 °C according to previous reports (Sato et al., 2005).Consequently, ~20% XX fish showed male characteristics and fertility, resulting in normal XX sex-reversed males, which was similar to results reported previously (Sato et al., 2005; Horie et al., 2016). All animal husbandry and experimentation were conducted in accordance with our Guide for Care and Use of Laboratory Animals and was approved by the Institutional Committee of Laboratory Animal Experimentation (University of Shizuoka, Shizuoka, Japan).

2.2. Induction of testis-ova differentiation

XY and XX sex-reversed males of Hd-rR medaka were kept in fixed dechlorinated water during the experiments. Using a static system, these medaka were immersed in dechlorinated water with 1200 ng/L, 800 ng/L, and 400 ng/L of EB (Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks and kept in dechlorinated water without EB for 2 weeks as described in our previous studies (Kinoshita et al., 2009; Hirakawa et al., 2012). In the present study, similar induction of testis-ova differentiation was recognized. The water in which medaka were immersed was replaced daily (every 24 h). Briefly, the water was prepared in new tanks daily, and medaka were transferred into new tanks. EB