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RESEARCH REPORT

Disruption of *dmrt1* rescues the all-male phenotype of the *cyp19a1a* mutant in zebrafish – a novel insight into the roles of aromatase/estrogens in gonadal differentiation and early folliculogenesis

Kun Wu, Weiyi Song, Zhiwei Zhang and Wei Ge*

ABSTRACT

Sex determination and differentiation are complex processes controlled by many different factors; however, the relationships among these factors are poorly understood. Zebrafish gonadal differentiation exhibits high plasticity involving multiple factors and pathways, which provides an excellent model for investigating the interactions between them. Ovarian aromatase (*cyp19a1a*) and *dmrt1* are key factors in directing vertebrate ovary and testis differentiation, respectively. Knockout of zebrafish *cyp19a1a* leads to all-male offspring, whereas the loss of *dmrt1* results in a female-biased sex ratio. In the present study, we established *dmrt1*$^{-/-}$; *cyp19a1a*$^{-/-}$ double mutant zebrafish and discovered that the introduction of the *dmrt1* mutation into the *cyp19a1a* mutant could rescue the all-male phenotype of the latter. Interestingly, despite the lack of aromatase/estrogens, the follicles in the ovary of the rescued *cyp19a1a* mutant could develop normally up to the previtellogenic stage. Further evidence suggested the ovarian aromatase directed ovarian differentiation by suppressing *dmrt1* expression via nuclear estrogen receptors (nERs). Our results provide solid evidence for an interaction between *cyp19a1a* and *dmrt1* in zebrafish gonadal differentiation, and for the dispensability of estrogens in controlling early folliculogenesis.

KEY WORDS: Dmrt1, Cyp19a1a, Aromatase, Sex differentiation, Folliculogenesis, Zebrafish

INTRODUCTION

Sex determination and differentiation are hot topics in developmental and reproductive biology. In mammals, sex differentiation is triggered by an upstream sex-determining factor *Sry*, which drives male development (Bachtrog et al., 2014; Cutting et al., 2013). Although the upstream sex determination signals vary in vertebrates, the downstream differentiation pathways tend to be more conserved (Devlin and Nagahama, 2002; Heule et al., 2014). With the development of new knockout technologies (Cong et al., 2013; Hwang et al., 2013), an increasing number of genes and their functions in gonadal differentiation have been assessed in different species, especially in zebrafish (Dranow et al., 2016; Lau et al., 2016; Lu et al., 2017; Nakamoto et al., 2018; Qin et al., 2018; Zhang et al., 2017, 2015). Unlike in mammals and some fish species, sex determination in domesticated zebrafish is considered to be a polygenic mechanism (Liew et al., 2012; Liew and Orban, 2014). The high plasticity of zebrafish gonadal development provides an excellent model for investigating the interactions between various regulatory factors.

Doublesex and mab-3-related transcription factor 1 (*dmrt1*) is a functionally important transcription factor in male differentiation across vertebrates (Eggers et al., 2014; Guo et al., 2005; Herpin and Schartl, 2011; Matson and Zarkower, 2012; Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009). Recent knockout studies in zebrafish also verified that *dmrt1* was necessary for zebrafish male differentiation (Lin et al., 2017; Webster et al., 2017). Aromatase, which is encoded by Cyp19 for the production of estrogens (Simpson et al., 1994), is well known to play a crucial role in fish sex differentiation (Nakamura et al., 2003, 1998; Paul-Prasanth et al., 2013; Takatsu et al., 2013). Knockout of the ovarian aromatase *cyp19a1a* but not the brain aromatase *cyp19a1b* in zebrafish resulted in an all-male phenotype (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017; Song et al., 2020).

The important roles of *dmrt1* and *cyp19a1a* in sexual development have been extensively investigated, but the knowledge about their actions and, in particular, their interactions in gonadal differentiation is still limited. In the present study, we first confirmed the essential role of *dmrt1* in driving male differentiation. Then we focused our attention on the potential interaction between the male-promoting *dmrt1* and female-promoting *cyp19a1a*. Surprisingly, we found that, in the absence of the *dmrt1* gene, the all-male phenotype of the *cyp19a1a* mutant was rescued with female follicles developing normally up to the previtellogenic (PV) stage (stage II) in double mutant fish (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$). Our discoveries suggest that Cyp19a1a plays a role in determining ovarian differentiation by suppressing *dmrt1* expression; however, the enzyme plays little role afterwards in early follicle development until the vitellogenic stage.

RESULTS AND DISCUSSION

Reverse of all-male phenotype of *cyp19a1a* mutant by *dmrt1* mutation

In this study, we selected a *dmrt1* null mutant fish line with a 7 bp deletion (*dmrt1*$^{-7/-7}$) (registered with ZFIN as umo15) for phenotype analysis because it is supposed to induce a frameshift mutation that disrupts Dmrt1 protein synthesis (Fig. S1). The *dmrt1*$^{-/-}$ mutants showed a significant female-biased phenotype (*dmrt1*$^{-/-}$, 88.8%±1.3; *dmrt1*$^{+/-}$, 42.6%±11.8; *dmrt1*$^{+/+}$, 50.8%±6.8; Fig. 1A) with normal folliculogenesis and fertility in females (Fig. S2). Additionally, all *dmrt1*$^{-/-}$ males were infertile because of failure to

Centre of Reproduction, Development and Aging (CRDA), Faculty of Health Sciences, University of Macau, Taipa, Macau 999078, China.

*Author for correspondence (weige@umac.mo/gezebrafish@gmail.com)

W.G., 0000-0002-4296-1585

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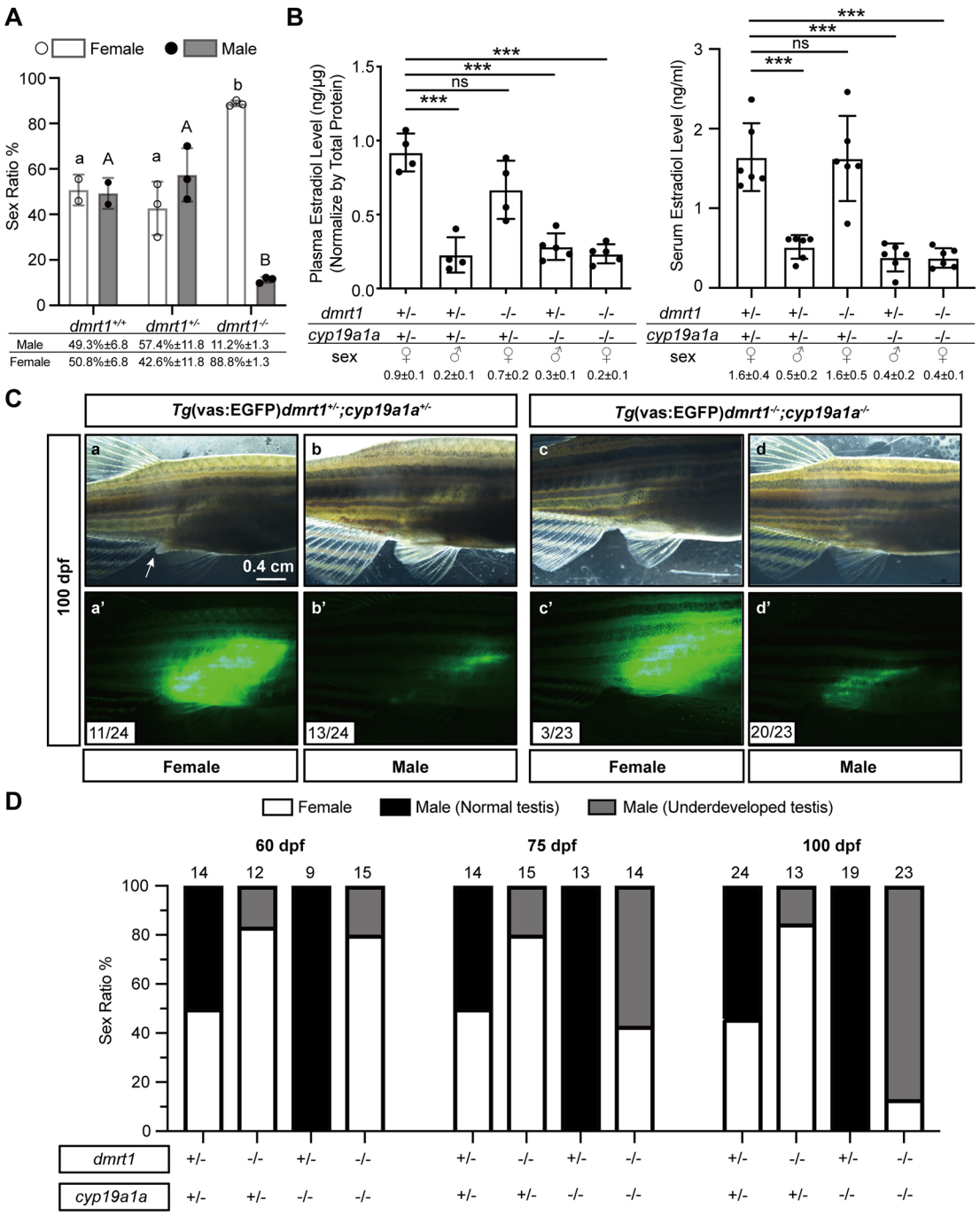

Fig. 1. Mutation of *dmrt1* rescues the all-male phenotype of *cyp19a1a* mutants. (A) Sex ratios in *dmrt1*$^{+/+}$, *dmrt1*$^{+/-}$ and *dmrt1*$^{-/-}$ siblings at 3-4 mpf (months post-fertilization). Data are mean±s.d. and the exact values are shown under the *x*-axis. Different letters (a and b or A and B) indicate statistical significance (P<0.01) revealed by one-way ANOVA and Tukey's test. (B) The plasma and serum estrogen levels in different genotypes of two separated batches of fish at 100 dpf. Data are mean±s.d. and the exact values are shown under the *x*-axis. Statistical significance was revealed by one-way ANOVA and Tukey's test (***P<0.001; ns, not significant). (C) The gross morphology of females (a,a′,c,c′) and males (b,b′,d,d′) in mutants [*Tg*(vas:EGFP)*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$] and controls [*Tg*(vas:EGFP)*dmrt1*$^{+/-}$;*cyp19a1a*$^{+/-}$] at 100 dpf. Arrow indicates genital papillae. The gonads with strong GFP signals were ovaries, whereas those with weak GFP signals were testes. (D) Sex ratios in different genotypes of *dmrt1*;*cyp19a1a* at 60 dpf, 75 dpf and 100 dpf.

induce female spawning and dysfunctional spermatogenesis (Fig. S3), which agrees well with recent similar studies in zebrafish (Lin et al., 2017; Webster et al., 2017) and the reported roles of DMRT1 in sex differentiation of other vertebrates (Matson et al., 2010, 2011; Smith et al., 2009). The female-biased sex ratio in *dmrt1* mutant, together with the all-male phenotype of *cyp19a1a* mutants (Lau et al., 2016; Yin et al., 2017), suggest that *cyp19a1a* and *dmrt1* may represent two major factors/pathways that drive female and male development, respectively. What will happen if both *cyp19a1a* and *dmrt1* are lost? To answer this question, we went on to generate a double mutant of these two genes (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$).

In our study, all the double mutants (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) exhibited masculinized secondary sexual characteristics, including the absence of genital papillae, a slimmer body and a brown anal fin

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(Fig. S4), making it difficult to distinguish their sex via appearance. In *dmrt1*$^{-/-}$ mutants, the fish could be sexed based on apparent morphological features (Webster et al., 2017). The lack of female sexual characteristics in the double mutants (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) might be due to insufficient estrogens (plasma, 0.2±0.1 ng/µg; serum, 0.4±0.1 ng/ml; Fig. 1B), which are essential for female characteristics (Brion et al., 2004).

To better identify the sex of *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ fish, we introduced the transgenic line *Tg*(vas:EGFP)^{zf45} into the double mutant line to generate *Tg*(vas:EGFP)*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ fish. According to previous reports from us and others (Krøvel and Olsen, 2004; Lau et al., 2016; Qin et al., 2018; Wang et al., 2007), the GFP signal is much higher in the ovary than in the testis and its intensity increases dramatically when the gonad differentiates into ovary. This feature allows live assessment of zebrafish sex during sex differentiation before sampling for histological confirmation. The GFP signals in control females (*dmrt1*$^{+/-}$;*cyp19a1a*$^{+/-}$) were strong at 100 dpf (days post-fertilization), in contrast to the weak signals in males. Surprisingly, some double mutant (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) individuals also showed strong GFP signals fully comparable with those in female controls (Fig. 1Ca′,c′), suggesting the formation of ovaries in the absence of *cyp1a91a*. Histological analysis confirmed the formation of well-developed ovaries containing many follicles (Fig. 2Bf,f′).

Analysis of sex ratios at 60, 75 and 100 dpf (Fig. 1D) showed that single *cyp19a1a*$^{-/-}$ mutants were all males (100%) at all time points examined, whereas majority of the *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants were females at 60 dpf (12 females/15, 80% females), which was comparable with that of single *dmrt1*$^{-/-}$ mutants (10 females/12, 83% females). Interestingly, at later times (75 and 100 dpf), the female ratio of the *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants decreased progressively (6 females/14, 43% females, 75 dpf; 3 females/23, 13% females, 100 dpf), while the ratio in *dmrt1*$^{-/-}$ mutants remained unchanged over time (12 females/15, 80% females, 75 dpf; 11 females/13, 85% females, 100 dpf). These results clearly indicate that disruption of *dmrt1* rescued the all-male phenotype of *cyp19a1a*$^{-/-}$ mutant zebrafish. However, the females in the *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants could not be maintained indefinitely and the fish likely underwent sex reversal from females to males. Further evidence is shown in the following histological analysis.

Novel insights on roles of *cyp19a1a*/estrogens in early folliculogenesis

To confirm the identity of female double mutants (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) revealed by GFP signal (see above), we further analyzed the same batch of fish at 75 dpf by histology. As we reported previously (Lau et al., 2016), all *cyp19a1a* single mutants (*dmrt1*$^{+/-}$;*cyp19a1a*$^{-/-}$) were males at 75 dpf (13 males/13, 0% females), in contrast to the control (*dmrt1*$^{+/-}$;*cyp19a1a*$^{+/-}$; 7 females/14, 50% females) and *dmrt1* single mutants (*dmrt1*$^{-/-}$;*cyp19a1a*$^{+/-}$; 12 females/15, 80% females). Surprisingly, the *dmrt1* and *cyp19a1a* double mutants (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) showed normal sex ratio (6 females/14, 43% females) comparable with that in the control group, and the females had well-developed ovaries (Fig. 2A). However, in contrast to the ovaries of control females that contained follicles of primary growth (PG), PV and early vitellogenic (EV) stages, the ovaries of the double mutants contained PG and PV follicles only, without any follicles entering vitellogenic growth, suggesting a blockade at the PV-EV transition (Fig. 2A,C). This result indicates clearly that all stages of early folliculogenesis occurred normally in double mutant ovaries, including PG follicle growth, PG-PV transition and development of full-sized PV follicles, and all these took place without the ovarian aromatase (*cyp19a1a*). This is surprising, as the ovarian aromatase Cyp19a1a has long been believed to play a crucial role in folliculogenesis, including early stages, as it is expressed specifically in the ovary at the time of gonadal differentiation and its expression level increases significantly at the PG-PV transition (Chen et al., 2017; Zhu et al., 2018). This traditional view on roles of aromatase in female development is obviously challenged by our finding with *cyp19a1a* and *dmrt1* double knockout.

To confirm whether the arrest of follicle development was due to a delayed PV-EV transition or permanent blockade, we also examined the ovaries of the double mutants at 100 dpf when the control fish were sexually mature and spawning. The results showed that the follicles in the double mutant ovaries (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) remained arrested at PV stage at 100 dpf (Fig. 2B,D). Furthermore, the PV follicles in the double mutants were significantly larger than those in the control ovaries (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$, 270.22±49.73 µm; *dmrt1*$^{+/-}$;*cyp19a1a*$^{+/-}$, 238.29±35.17 µm; Fig. 2E), still without any vitellogenin accumulation. The mechanism underlying the PV-EV blockade in the ovary of the double mutant (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$) is unknown, and it will be an interesting issue for further studies. One possible reason is that, without estrogens, the liver would not produce vitellogenin, the precursor of the yolk mass in vitellogenic oocytes (Lubzens et al., 2010; Nagahama and Yamashita, 2008). Our data on plasma and serum estradiol levels also support this hypothesis (Fig. 1B). In mice, although vitellogenesis does not occur, follicular maturation is disrupted and ovulation failure is observed in the absence of aromatase (Fisher et al., 1998). Our evidence indicates that, although estrogens play little role in early folliculogenesis in zebrafish, they are crucial for late stage of follicle development and maturation, consistent with the situation in medaka (Nakamoto et al., 2018) and mammals (Fisher et al., 1998).

In medaka fish, although the *cyp19a1a* mutant could also form an ovary in the same way as the zebrafish double mutant (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$), the female mutant eventually underwent sex reversal to become male, suggesting an important role for estrogens in maintaining the ovary (Nakamoto et al., 2018). We also observed similar phenomenon. The follicles in *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ mutant zebrafish exhibited morphological features of atresia after 3 months, such as membrane disintegration, follicle cell proliferation and zona radiata breakdown (Fig. S5) (Morais et al., 2012; Ucuncu and Cakici, 2009). The follicles at 100 dpf gradually underwent degeneration with interfollicular spaces becoming slowly occupied by stromal cells (Fig. 2Bf-f‴; Fig. S6a-a‴), a typical feature seen during the transition from juvenile ovary to testis (Hill and Janz, 2003; Uchida et al., 2002) and in the underdeveloped testes of *dmrt1*$^{-/-}$ males (Fig. 2Bg,g′). The follicle degeneration occurred slowly, as evidenced by different degrees of follicle degeneration observed at 100 dpf in *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants, followed by sex reversal to testis (Fig. S6). However, similar to the testis in *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutant males (Fig. 2Bg,g′; Fig. S6d-d‴), the testis development following ovarian degeneration could not proceed further due to the lack of *dmrt1* (Fig. S6). This female-to-male conversion explains the progressive decrease in female-to-male ratio over time (Fig. 1D).

Interaction between *cyp19a1a*/estrogens and *dmrt1* in gonadal differentiation

The rescue of the all-male phenotype of *cyp19a1a* single mutants by *dmrt1* mutation raised an interesting question about the regulatory

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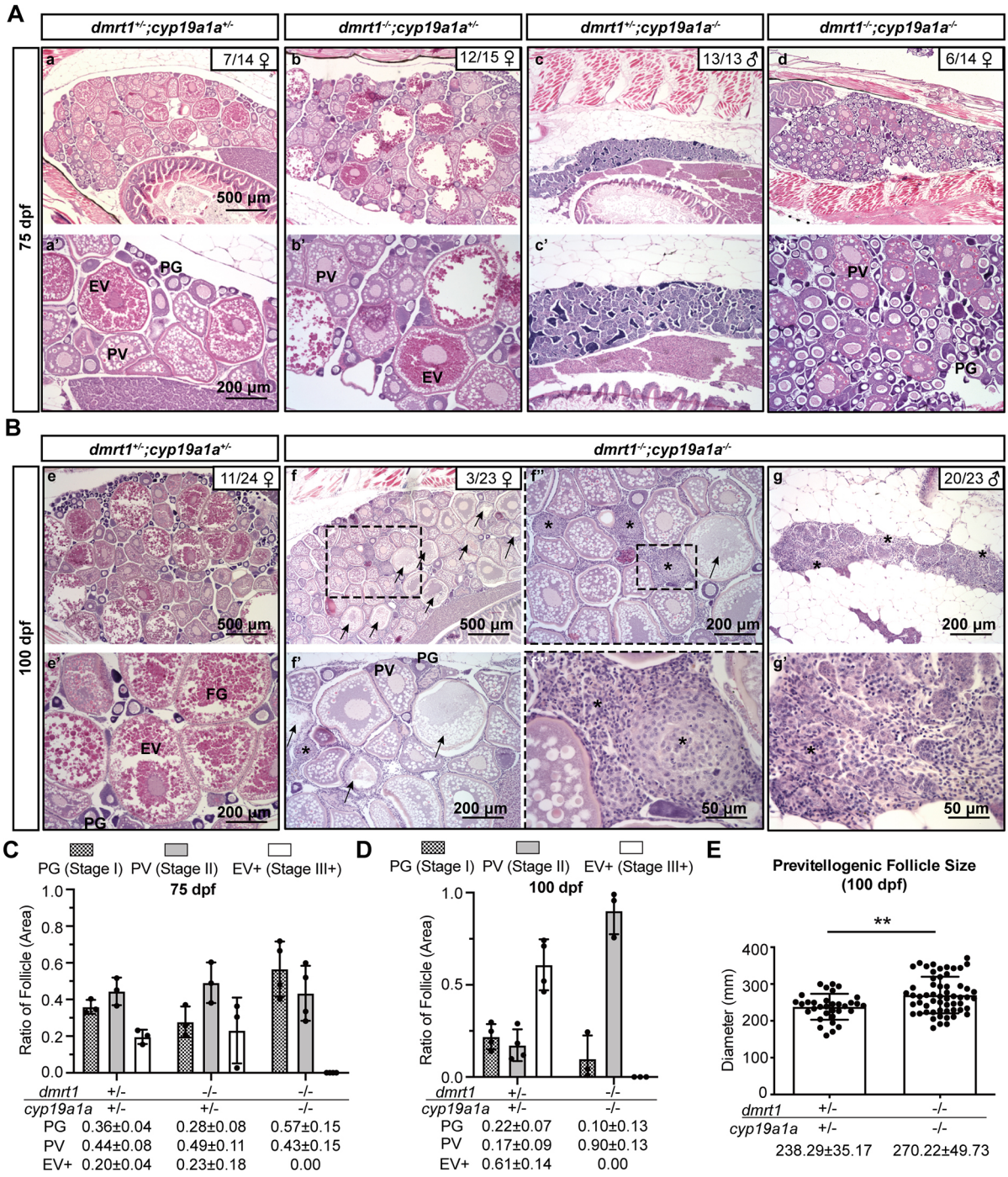

Fig. 2. Phenotype analysis of female zebrafish in different genotypes. (A,B) Histological examination of gonads in different genotypes of *dmrt1;cyp19a1a* at 75 dpf (A) and 100 dpf (B). PG, primary growth; PV, pre-vitellogenic; EV, early vitellogenic; FG, full grown. Arrows indicate atresic/degenerating follicles. Asterisks indicate stromal cells. (C,D) Ratios of PG, PV and vitellogenic (EV+) follicle areas in different genotypes at 75 dpf (C) and 100 dpf (D). Data are mean±s.d. The exact values are shown under the *x*-axis. (E) The size of PV follicles in controls and double mutants ($dmrt1^{-/-}$;$cyp19a1a^{-/-}$) at 100 dpf. Each dot represents the average diameter of PV follicles with visible germinal vesicles from each fish (quantified from three different sections). Data are mean±s.d. and the exact values are shown under the *x*-axis. Statistical significance was revealed by two-tailed unpaired Student's *t*-test (**P<0.01).

relationship between *dmrt1* and *cyp19a1a*. To address this question, we analyzed the expression levels of *dmrt1* and *cyp19a1a* in wild-type juvenile zebrafish before sex differentiation. At 15 dpf, which is 10 days before sex differentiation (25 dpf) (Chen and Ge, 2013), the expression levels of *dmrt1* and *cyp19a1a* showed the opposite relationship. The individuals with high *dmrt1* expression showed lower expression of *cyp19a1a*, and vice versa (Fig. 3A). Further evidence suggested that the reciprocal relationship of *dmrt1* and *cyp19a1a* could be due to an inhibition of *dmrt1* expression by estrogens produced by Cyp19a1a. First, we examined *dmrt1* expression in the control ($cyp19a1a^{+/-}$) and *cyp19a1a* mutant ($cyp19a1a^{-/-}$) at 25 dpf. As shown in Fig. 3B, two groups of fish could be identified in the presence of Cyp19a1a ($cyp19a1a^{+/-}$) at 25 dpf when gonadal differentiation occurs, namely, the *dmrt1* high

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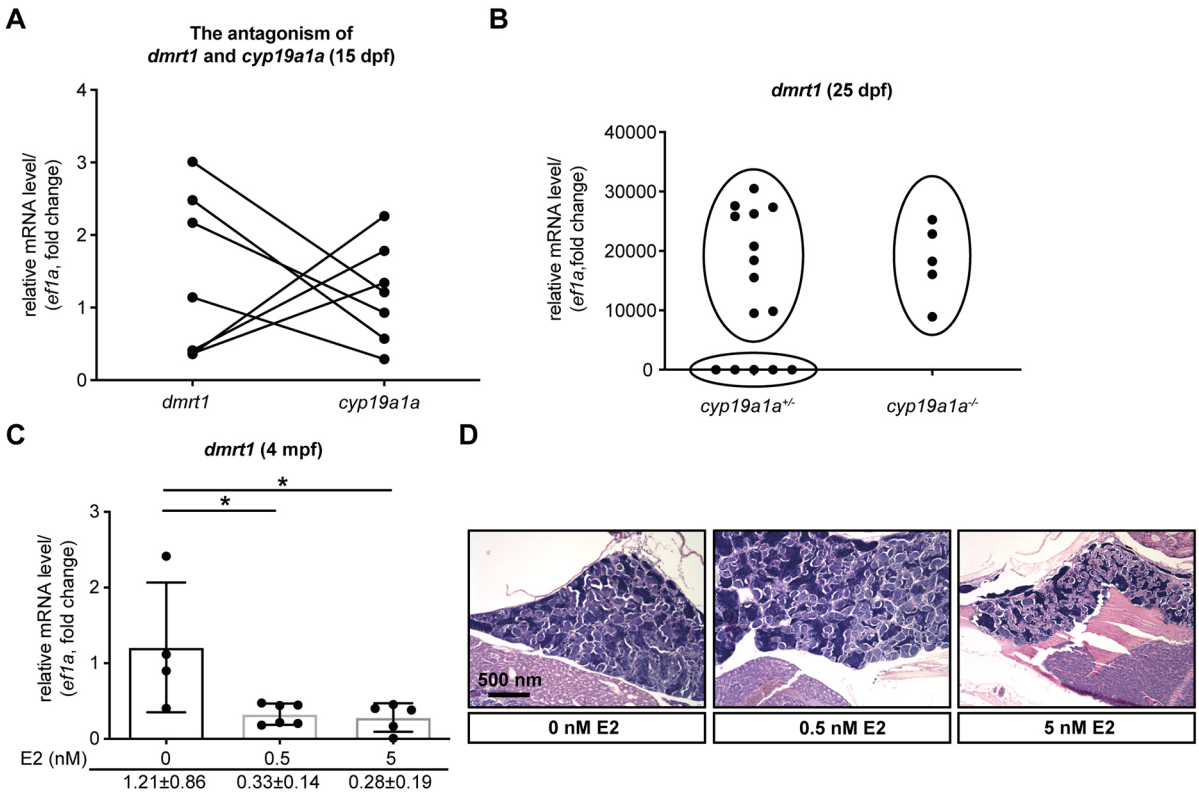

Fig. 3. Estrogen suppression of *dmrt1* expression. (A) The reciprocal expression of *dmrt1* and *cyp19a1a* in wild-type individuals at 15 dpf (n=7). (B) Expression of *dmrt1* in *cyp19a1a*$^{+/-}$ (n=15) and *cyp19a1a*$^{-/-}$ (n=5) zebrafish at 25 dpf. Individual data points are shown. (C) Response of *dmrt1* expression to E2 treatment (0 nM, 1.21±0.86, n=4; 0.5 nM, 0.33±0.14, n=6; 5 nM, 0.28±0.19, n=6) at 4 mpf. The data points are expressed as mean±s.d. and the exact values are shown under the x-axis. Statistical significance was revealed by one-way ANOVA and Tukey's test (*P<0.05). (D) Histology of representative fish exposed to different concentrations of E2 (0 nM, 0.5 nM and 5 nM).

expressers and low expressers. In contrast, all *cyp19a1a* mutants (*cyp19a1a*$^{-/-}$) showed high expression of *dmrt1*, likely owing to the lack of estrogens that suppress *dmrt1* expression. Second, exposure of male adult zebrafish to estradiol at 0.5 and 5 nM significantly reduced the levels of *dmrt1* expression in mature testes (0.5 nM, 0.33 ±0.14; 5 nM, 0.28±0.19; 0 nM, 1.21±0.86; Fig. 3C), although the treatment did not cause any obvious changes in testes at the histological level (Fig. 3D). The reciprocal relationship between *dmrt1* and *cyp19a1a* is also supported by recent reports that DNA methylation of *dmrt1* and *cyp19a1a* were inversely correlated in developing gonads (Piferrer et al., 2019; Todd et al., 2019).

Receptor mechanism of estradiol action in suppressing *dmrt1* expression

The results described above strongly suggest a role for *cyp19a1a*, via estrogens, in suppressing *dmrt1* expression in female individuals, thereby driving ovarian differentiation. To understand how estrogens work in the process, we performed a comprehensive genetic analysis, making use of zebrafish mutants of both *cyp19a1a* and nuclear estrogen receptors (nERs; *esr1*, *esr2a* and *esr2b*), which we generated and reported recently (Lau et al., 2016; Lu et al., 2017).

The fish were sampled at 75 dpf after gonadal differentiation (normally 25-30 dpf); therefore, the sex ratios recorded reflected mostly the maintenance of femaleness rather than female differentiation. As shown in Fig. 4A, the sex ratio of control fish was ∼50% for males and females at 75 dpf. Some individuals in *esr1*$^{-/-}$;*esr2a*$^{-/-}$ double mutants (*esr1* and *esr2a* genes are closely linked on the same chromosome) or *esr2b*$^{-/-}$ single mutants could develop into females with female ratios relatively lower than the control, which is similar to our recent report (Lu et al., 2017). All fish were males in the presence of *dmrt1* but absence of aromatase (*cyp19a1a*$^{-/-}$), and most fish examined were also males in the triple knockout of all three nERs (*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) (16/18) with two fish showing intersexuality undergoing female-to-male sex reversal. The all-male phenotype in the triple knockout of nERs could be completely reversed to 100% females by deleting *dmrt1* gene (*dmrt1*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$). Consistently, quadruple knockout of aromatase and all nERs (*cyp19a1a*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) also resulted in all-male phenotype. Interestingly, disruption of *dmrt1* reversed the sex ratio from 100% males in the quadruple knockout of aromatase and nERs (*cyp19a1a*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) to 100% females in the quintuple knockout (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$). Histological analysis showed that the ovaries could form in the quintuple knockout, and the follicles could also develop up to PV stage without aromatase and estrogen receptors (Fig. 4B). Our data using multiple mutants ranging from single (*dmrt1*$^{-/-}$, *cyp19a1a*$^{-/-}$ and *esr2b*$^{-/-}$) to quintuple mutant (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) clearly demonstrate the involvement of nERs in mediating estrogen suppression of *dmrt1* expression.

In summary, using a series of zebrafish mutant lines, especially *dmrt1*$^{-/-}$ and *cyp19a1a*$^{-/-}$ mutants, we provide clear evidence that the ovarian aromatase *cyp19a1a* works to drive ovarian differentiation by suppressing *dmrt1*, which is initially expressed in all individuals before sex differentiation to direct gonadal development towards the testis. Further experiments using estrogen receptor mutants (*esr1*$^{-/-}$, *esr2a*$^{-/-}$ and *esr2b*$^{-/-}$) demonstrated

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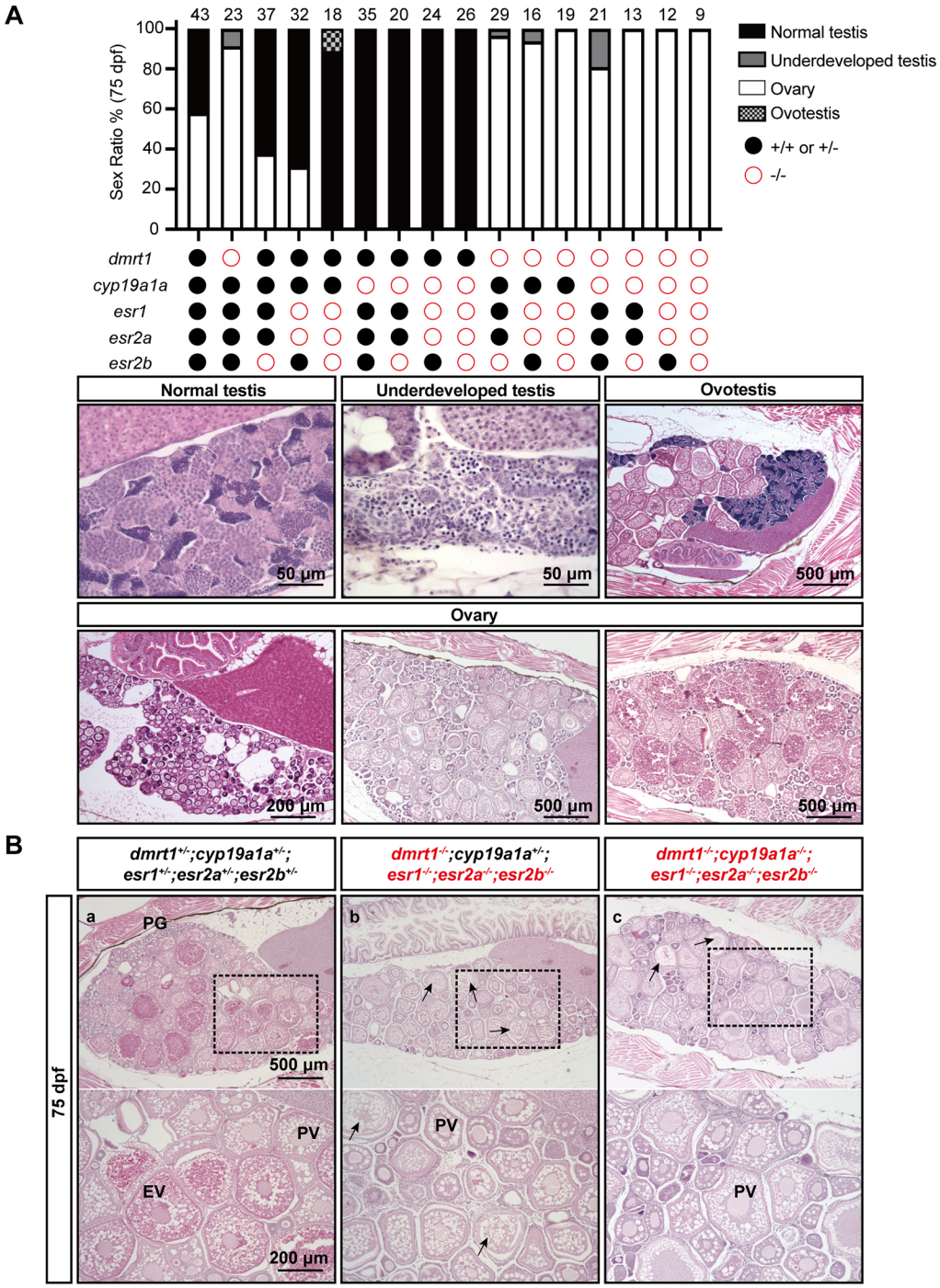

Fig. 4. Disrupting all the estrogen receptors in *dmrt1* mutants. (A) Sex ratios at 75 dpf in different mutation combinations of five genes (*dmrt1*, *cyp19a1a*, *esr1*, *esr2a* and *esr2b*). The data were pooled from fish of five independent breedings to enlarge the sample size. (B) Representative histological sections corresponding to different gonadal types, including the ovaries in heterozygous control, quadruple knockout (*dmrt1*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) and quintuple knockout (*dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$;*esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) at 75 dpf (*n*=9-43 for each genotype). Arrows indicate atresic/degenerating follicles. PV, pre-vitellogenic; EV, early vitellogenic.

involvement of nERs in producing estrogen-mediated suppression of *dmrt1* expression. Furthermore, the present study provides crucial insight into the role of estrogens in controlling early folliculogenesis. In contrast to the traditional view on estrogen involvement in folliculogenesis, our data show that after knocking down the expression of *dmrt1* at gonadal differentiation stage in females, the ovarian aromatase no longer plays any significant roles in promoting early follicle development, including follicle activation (Fig. 5). Further studies on the regulatory mechanisms upstream of *cyp19a1a* will provide insight into the mechanisms underlying zebrafish sex determination. By generating mutants with mutations of up to five genes, this study also demonstrates the power of zebrafish model in genetic analysis.

MATERIALS AND METHODS

Animals

The following alleles generated by our previous studies (Lau et al., 2016; Lu et al., 2017) were used in the present study: *cyp19a1a*^{umo5}, *esr1*^{umo10}; *esr2a*^{umo12} and *esr2b*^{umo13} (see Table S1 for allele information). The alleles mentioned above and the *dmrt1* mutant line (registered with ZFIN as *dmrt1*^{umo15}; Fig. S1) generated and used in the present study were based on the AB zebrafish strain (China Zebrafish Resource Center, Wuhan, China). The transgenic line *Tg*(*vas:*EGFP)^{zf45} (Krøvel and Olsen, 2002) was generously provided by Dr. Rüdiger Schulz (Utrecht University, The Netherlands). Wild-type (+/+) and/or heterozygotes (+/−) zebrafish were used as controls in the present study. All experiments were performed according to the guidelines and regulations approved by the Research Ethics Committee of the University of Macau (AEC-13-002).

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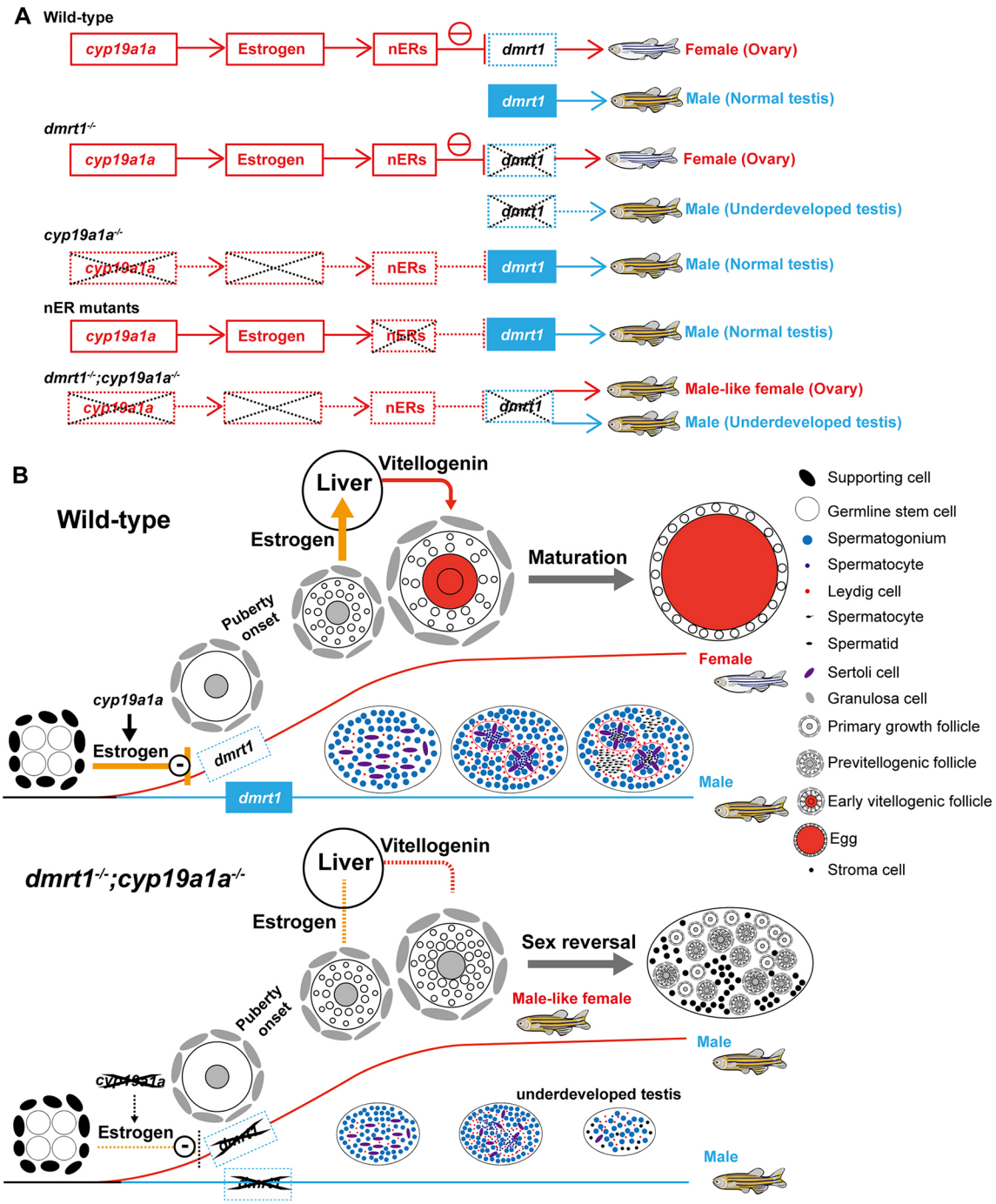

Fig. 5. Schematic model on roles of estrogens in sex determination and differentiation in zebrafish. (A) Mechanism of estrogen signaling and action in zebrafish sex differentiation. The ovarian aromatase (*cyp19a1a*) plays a crucial role via nuclear estrogen receptors (nERs) in driving ovarian differentiation by suppressing the expression of *dmrt1*, which by default directs all individuals towards males. (B) Roles of estrogens in zebrafish ovarian differentiation and folliculogenesis. In wild-type fish, *dmrt1* is expressed in all individuals to promote gonadal differentiation towards testis. In genetic females, the ovarian aromatase *cyp19a1a* suppresses *dmrt1* expression, therefore directing the gonads to differentiate into ovaries. Although Dmrt1 continues to play a crucial role in supporting spermatogenesis after gonadal differentiation, estrogens surprisingly become dispensable for early follicle development, including PG follicle growth, PG-PV transition and PV follicle development. In *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants, the ovaries are well formed but the follicle development is eventually blocked at the PV-EV transition after PV follicles grow to their full sizes and before vitellogenic growth starts. This is likely due to the lack of vitellogenin production in the liver, which is well known to be estrogen dependent. Despite the dispensability of estrogens in early folliculogenesis, they are crucial in maintaining the femaleness. In *dmrt1*$^{-/-}$;*cyp19a1a*$^{-/-}$ double mutants, the ovaries with PV follicles gradually regress after about 3 months and convert into testis-like structures; however, spermatogenesis is impaired in the absence of Dmrt1.

Fish husbandry

The fish were raised in an environmental chamber (Thermo Fisher Scientific) and flow-through aquarium system (Tecniplast) at 28±1°C with a photoperiod of 14 hours of light and 10 hours of dark. The larvae were fed with paramecia and artemia, and the adults were fed with artemia and commercial dry food.

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Generation of *dmrt1* null zebrafish mutant line(s)

The CRISPR/Cas9 approach was used to establish the *dmrt1* mutant in zebrafish according to the protocols reported previously (Hwang et al., 2013; Jao et al., 2013; Lau et al., 2016; Lu et al., 2017). An sgRNA (oligo sequence shown in Table S2) targeting exon II of *dmrt1* was designed via online websites (http://zifit.partners.org/) to avoid potential off-target effects. The gRNA and Cas9 mRNA were generated through *in vitro* transcription from DraI-digested pDR274 (Addgene Plasmid #42250) and pCS2-nCas9n (Addgene Plasmid #47929) using the mMACHINE T7 and mMACHINE SP6 kit (Invitrogen) according to the manufacturer's instructions. Then, 4.6 nl of a mixture with gRNA (75 ng/µl) and Cas9 (200 ng/µl) mRNA was injected into one- or two-cell-stage embryos by the Drummond Nanoject system (Drummond Scientific). The F0 mutants were screened using a high resolution melting analysis (HRMA) and a heterozygous mobility assay (HMA), followed by sequencing confirmation (Dahlem et al., 2012; Ota et al., 2013; Zhang et al., 2015). The F0 founders carrying mosaic mutations were mated with the wild-type partners to obtain heterozygous F1(+/−). Sibling female and male F1 individuals carrying the same mutation were mated to obtain the homozygous F2 (−/−).

Three different mutant lines with sequence deletions (−3, −6 and −7) were generated. The CRISPR targeting site and the mutated sequences are shown in Fig S1. The mutant line with a seven-base pair deletion [*dmrt1* (−7)] was chosen and maintained for phenotype analysis because it is supposed to induce a frameshift mutation that disrupts Dmrt1 protein synthesis.

Genotyping of mutants

Genomic DNA from each individual embryo or a small piece of the caudal fin was extracted by alkaline lysis (Zhang et al., 2015). NaOH (30-50 µl, 50 nmol/µl) was used to extract the genomic DNA at 95°C for 10 min. Then, 3-5 µl Tris-HCl (pH 8.0) was added to the solution to neutralize the reaction system. The genomic DNA was used for HRMA with Precision Melt Analysis software (Bio-Rad) to analyze the difference of the melt curves for distinguishing different genotypes (Dahlem et al., 2012). The primers used for HRMA are listed in Table S2.

Sampling, photographing and histological examination

The fish were sampled at different time points for phenotype analysis. Histological examination was performed on paraffin sections using Hematoxylin and Eosin (H&E) staining. The fish were sacrificed using MS-222 (Sigma-Aldrich). The gross morphology of each fish was photographed with a digital camera (Canon EOS 7D). The anal fin and cloaca were viewed using a Nikon SMZ18 anatomic microscope and photographed with the Digit Sight DS-Fi2 digital camera (Nikon). Fluorescent images were acquired on the Nikon SMZ18 microscope with a Nikon INTRNSLINGHT C-HGFI laser and photographed with the Digit Sight DS-Fi2 digital camera.

For histological analysis, the entire fish was fixed in Bouin's solution for at least 24 h. Dehydration and infiltration were then performed on the ASP6025S Automatic Vacuum Tissue Processor (Leica). The samples were embedded with paraffin, followed by serial sectioning at 5 µm. The sections were stained with H&E and viewed on the Nikon ECLIPSE Ni-U microscope. The photos were taken using the Digit Sight DS-Fi2 digital camera. Sibling wild-type (+/+) and/ or heterozygous (+/−) fish were used as controls for phenotype analysis.

Fertility assessment

Female fertility was assessed by the number of eggs ovulated and the percentage of survived embryos. The female fish (+/− or −/−) were naturally mated with wild-type (+/+) male fish, separately. The number of ovulated eggs was counted after spawning. The number of survived embryos was counted 24 h after spawning. The number of eggs ovulated and the embryo survival rate for the female mutants ($dmrt1^{-/-}$) were similar to wild-type females ($dmrt1^{+/+}$) and the female mutants ($dmrt1^{-/-}$) were therefore considered fertile.

Male fertility was assessed by mating performance, which is defined according to their ability to stimulate spawning of the wild-type (+/+) females. At least five individual male fish of each genotype (+/+ or −/−) were tested with the wild-type (+/+) female fish separately. The tests were repeated at 5-day intervals. The spawning rate was defined as the ratio of successful spawning pairs over total pair number. The experiment was repeated at least four times.

Follicle stages

Follicles were staged according to morphological and size criteria as we reported previously (Chen and Ge, 2013; Wang and Ge, 2004). Follicle development was staged as follows: PG (primary growth, stage I), PV (previtellogenic, stage II), EV (early vitellogenic, early stage III), MV (mid-vitellogenic, mid-stage III), LV (late-vitellogenic, late stage III) and FG (full-grown).

Sex identification of zebrafish

In general, the gender of zebrafish was identified according to dimorphic morphological features, such as body shape, fin color and genital papillae (Brion et al., 2004), and, if necessary, by dissection under a Nikon SMZ18 stereomicroscope (Nikon, Japan) and/or histological examination. To monitor gonadal differentiation real-time without sacrificing the fish, we introduced the transgene *Tg*(vas:EGFP)^{zf45} into the mutants. As reported previously by us and others, the intensity of GFP fluorescence increased dramatically in the gonads when they differentiate towards ovaries (Lau et al., 2016; Qin et al., 2018; Wang et al., 2007). The sex identity was confirmed further by histology at the end of sampling. In counting the sex of double mutant fish ($dmrt1^{-/-}$;$cyp19a1a^{-/-}$), only the fish with well-formed ovaries in section were taken as females (Fig. 2Bf-f‴; Fig. S6a-a‴) while the fish with severely degenerating ovaries (Fig. S6b-b‴,c-c‴) or underdeveloped testes (Fig. 2Bg,g′; Fig. S6d-d‴) were taken as males.

For different combinations of quintuple mutants (Fig. 4), fish were firstly separated by genotype. For controls and $dmrt1^{-/-}$ single mutants, the fish were sexed according to obvious dimorphic morphological features (Webster et al., 2017). For $cyp19a1a^{-/-}$ single mutants, all fish were males according to our previous report. For other genotypes, all fish were sexed by histological sectioning and analysis.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from juvenile fish (whole fish) and adult fish testes (isolated gonads) using TRIzol (Invitrogen) according to the manufacturer's protocol. Then, reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed on the CFX384 Real-Time System (Bio-Rad) using primers listed in Table S2. Melting curve analysis was performed to demonstrate primer specificity. A standard curve was included in each real-time PCR assay for quantification. The relative gene expression level was normalized to the level of *ef1a* and expressed as a fold change compared with the control group.

Measurement of E2 levels

The fish were raised until 100 dpf when the body size was large enough for blood collection. In this study, both plasma and serum E2 levels were assessed, and two different batches of fish were used for collecting and preparing plasma and serum. Each fish was anesthetized using MS-222 before sacrifice (Sigma-Aldrich). Once completely anesthetized, the fish blood samples were collected from the heart with a glass capillary or a 10 µl tip and transferred into heparin-sodium-rinsed tubes. The supernatants (plasma) were collected after centrifugation [3000 rpm (850 ***g***), 30 min, 4°C] and then the protein concentrations were quantified using a 2-D Quant Kit (GE Healthcare). The level of E2 in the plasma was measured using an ELISA Kit (Neogen Corporation) according to the manufacturer's instructions.

To measure E2 level in the serum, the blood samples were transferred into 1.5 ml tubes without heparin-sodium treatment. Then the samples were kept at room temperature for 1 h to separate the serum. The supernatants (serum) were collected after centrifugation [3000 rpm (850 ***g***), 30 min, 4°C]. The level of E2 in the serum was measured using an ELISA Kit (Neogen Corporation), according to the manufacturer's instructions.

Continuous E2 treatment

Adult wild-type fish were treated with E2 (0, 0.5 and 5 nM) for 20 days (from 100 dpf to 120 dpf) continuously. Briefly, 30 fish were divided into

three groups, and each group with 10 fish was placed in a 6 liter tank containing E2 at different concentrations. The E2 stock was prepared with ethanol and the same volume of ethanol was added to the control group. The water was changed with new E2 every day.

Data analysis

All values are presented as the mean±s.d., and the significance (*P<0.05; **P<0.01; ***P<0.001; ns, not significant) of the data was analyzed by Student's t-test or one-way ANOVA using Prism 8 (GraphPad) on an Apple Mac.

No statistical method was applied to predetermine sample size. The sample size of control and mutant zebrafish used was according to experimental considerations. For each experiment, sample size is indicated in the relevant figure or figure legend. The sample size reflects the number of independent biological replicates. All experiments were performed at least twice.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.G.; Methodology: K.W., W.S., Z.Z.; Software: K.W., W.S.; Validation: K.W.; Formal analysis: K.W., W.S., W.G.; Investigation: K.W., W.S.; Resources: Z.Z.; Data curation: K.W., W.S.; Writing - original draft: K.W.; Writing - review & editing: K.W., W.G.; Visualization: K.W.; Supervision: W.G.; Project administration: K.W., W.G.; Funding acquisition: W.G.

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Supplementary information

Supplementary information available online at
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References

Bachtrog, D., Mankable s, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T. L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R. et al. (2014). Sex determination: why so many ways of doing it? *PLoS Biol.* **12**, e1001899. doi:10.1371/journal.pbio.1001899

Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J. and Flammarion, P. (2004). Impacts of 17 beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (Danio rerio). *Aquat. Toxicol.* **68**, 193-217. doi:10.1016/j.aquatox.2004.01.022

Chen, W. and Ge, W. (2013). Gonad differentiation and puberty onset in the zebrafish: evidence for the dependence of puberty onset on body growth but not age in females. *Mol. Reprod. Dev.* **80**, 384-392. doi:10.1002/mrd.22172

Chen, W., Liu, L. and Ge, W. (2017). Expression analysis of growth differentiation factor 9 (Gdf9/gdf9), anti-müllerian hormone (Amh/amh) and aromatase (Cyp19a1a/cyp19a1a) during gonadal differentiation of the zebrafish, Danio rerio. *Biol. Reprod.* **96**, 401-413. doi:10.1095/biolreprod.116.144964

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A. et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823. doi:10.1126/science.1231143

Cutting, A., Chue, J. and Smith, C. A. (2013). Just how conserved is vertebrate sex determination? *Dev. Dyn.* **242**, 380-387. doi:10.1002/dvdy.23944

Dahlem, T. J., Hoshijima, K., Jurynec, M. J., Gunther, D., Starker, C. G., Locke, A. S., Weis, A. M., Voytas, D. F. and Grunwald, D. J. (2012). Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *Plos Genetics* **8**, e1002861. doi:10.1371/journal.pgen.1002861

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191-364. doi:10.1016/S0044-8486(02)00057-1

Dranow, D. B., Hu, K., Bird, A. M., Lawry, S. T., Adams, M. T., Sanchez, A., Amatruda, J. F. and 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. doi:10.1371/journal.pgen.1006323

Eggers, S., Ohnesorg, T. and Sinclair, A. (2014). Genetic regulation of mammalian gonad development. *Nat. Rev. Endocrinol.* **10**, 673-683. doi:10.1038/nrendo.2014.163

Fisher, C. R., Graves, K. H., Parlow, A. F. and Simpson, E. R. (1998). Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc. Natl. Acad. Sci. USA* **95**, 6965-6970. doi:10.1073/pnas.95.12.6965

Guo, Y., Cheng, H., Huang, X., Gao, S., Yu, H. and Zhou, R. (2005). Gene structure, multiple alternative splicing, and expression in gonads of zebrafish Dmrt1. *Biochem. Biophys. Res. Commun.* **330**, 950-957. doi:10.1016/j.bbrc.2005.03.066

Herpin, A. and Schartl, M. (2011). Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish. *FEBS J.* **278**, 1010-1019. doi:10.1111/j.1742-4658.2011.08030.x

Heule, C., Salzburger, W. and Böhne, A. (2014). Genetics of sexual development: an evolutionary playground for fish. *Genetics* **196**, 579-591. doi:10.1534/genetics.114.161158

Hill, R. L., Jr and Janz, D. M. (2003). Developmental estrogenic exposure in zebrafish (Danio rerio): I. Effects on sex ratio and breeding success. *Aquat. Toxicol.* **63**, 417-429. doi:10.1016/S0166-445X(02)00207-2

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J.-R. J. and Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227-229. doi:10.1038/nbt.2501

Jao, L. E., Wente, S. R., and Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc. Natl Acad. Sci. USA* **110**, 13904-13909. doi:10.1073/pnas.1308335110

Krøvel, A. V. and Olsen, L. C. (2002). Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish. *Mech. Dev.* **116**, 141-150. doi:10.1016/S0925-4773(02)00154-5

Krøvel, A. V. and Olsen, L. C. (2004). Sexual dimorphic expression pattern of a splice variant of zebrafish vasa during gonadal development. *Dev. Biol.* **271**, 190-197. doi:10.1016/j.ydbio.2004.04.004

Lau, E. S.-W., Zhang, Z., Qin, M. and 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. doi:10.1038/srep37357

Liew, W. C. and Orban, L. (2014). Zebrafish sex: a complicated affair. *Brief. Funct. Genomics* **13**, 172-187. doi:10.1093/bfgp/elt041

Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R. and Orban, L. (2012). Polygenic sex determination system in zebrafish. *PLoS ONE* **7**, e34397. doi:10.1371/journal.pone.0034397

Lin, Q., Mei, J., Li, Z., Zhang, X., Zhou, L. and Gui, J.-F. (2017). Distinct and cooperative roles of amh and dmrt1 in self-renewal and differentiation of male germ cells in zebrafish. *Genetics* **207**, 1007-1022. doi:10.1534/genetics.117.300274

Lu, H., Cui, Y., Jiang, L. and Ge, W. (2017). Functional analysis of nuclear estrogen receptors in zebrafish reproduction by genome editing approach. *Endocrinology* **158**, 2292-2308. doi:10.1210/en.2017-00215

Lubzens, E., Young, G., Bobe, J. and Cerdà, J. (2010). Oogenesis in teleosts: how fish eggs are formed. *Gen. Comp. Endocrinol.* **165**, 367-389. doi:10.1016/j.ygcen.2009.05.022

Matson, C. K. and Zarkower, D. (2012). Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. *Nat. Rev. Genet.* **13**, 163-174. doi:10.1038/nrg3161

Matson, C. K., Murphy, M. W., Griswold, M. D., Yoshida, S., Bardwell, V. J. and Zarkower, D. (2010). The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. *Dev. Cell* **19**, 612-624. doi:10.1016/j.devcel.2010.09.010

Matson, C. K., Murphy, M. W., Sarver, A. L., Griswold, M. D., Bardwell, V. J. and Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* **476**, 101-104. doi:10.1038/nature10239

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N. et al. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **417**, 559-563. doi:10.1038/nature751

Morais, R. D. V. S., Thomé, R. G., Lemos, F. S., Bazzoli, N. and Rizzo, E. (2012). Autophagy and apoptosis interplay during follicular atresia in fish ovary: a morphological and immunocytochemical study. *Cell Tissue Res.* **347**, 467-478. doi:10.1007/s00441-012-1327-6

Nagahama, Y. and Yamashita, M. (2008). Regulation of oocyte maturation in fish. *Dev. Growth Differ.* **50**, S195-S219. doi:10.1111/j.1440-169X.2008.01019.x

Nakamoto, M., Shibata, Y., Ohno, K., Usami, T., Kamei, Y., Taniguchi, Y., Todo, T., Sakamoto, T., Young, G., Swanson, P. et al. (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. doi:10.1016/j.mce.2017.07.013

Nakamura, M., Kobayashi, T., Chang, X.-T. and Nagahama, Y. (1998). Gonadal sex differentiation in teleost fish. *J. Exp. Zool.* **281**, 362-372. doi:10.1002/(SICI)1097-010X(19980801)281:5<362::AID-JEZ3>3.0.CO;2-M

Nakamura, M., Bhandari, R. K. and Higa, M. (2003). The role estrogens play in sex differentiation and sex changes of fish. *Fish Physiol. Biochem.* **28**, 113-117. doi:10.1023/B:FISH.0000030495.99487.17

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A. et al. (2002). A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. *Proc. Natl. Acad. Sci. USA* **99**, 11778-11783. doi:10.1073/pnas.182314699

Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T. J., Grunwald, D. J., Okada, Y. and Kawahara, A. (2013). Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. *Genes Cells* **6**, 450-458. doi:10.1111/gtc.12050

Paul-Prasanth, B., Bhandari, R. K., Kobayashi, T., Horiguchi, R., Kobayashi, Y., Nakamoto, M., Shibata, Y., Sakai, F., Nakamura, M. and Nagahama, Y. (2013). Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists. *Sci. Rep.* **3**, 2862. doi:10.1038/srep02862

Piferrer, F., Anastasiadi, D., Valdivieso, A., Sánchez-Baizán, N., Moraleda-Prados, J. and Ribas, L. (2019). The model of the conserved epigenetic regulation of sex. *Front. Genet.* **10**, 857. doi:10.3389/fgene.2019.00857

Qin, M., Zhang, Z., Song, W., Wong, Q. W.-L., Chen, W., Shirgaonkar, N. and Ge, W. (2018). Roles of Figla/figla in juvenile ovary development and follicle formation during zebrafish gonadogenesis. *Endocrinology* **159**, 3699-3722. doi:10.1210/en.2018-00648

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y. J., Fisher, C. R., Michael, M. D. et al. (1994). Aromatase cytochrome-p450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15**, 342-355. doi:10.1210/edrv-15-3-342

Smith, C. A., Roeszler, K. N., Ohnesorg, T., Cummins, D. M., Farlie, P. G., Doran, T. J. and Sinclair, A. H. (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature* **461**, 267-271. doi:10.1038/nature08298

Song, W., Lu, H., Wu, K., Zhang, Z., Shuk-Wa Lau, E. and Ge, W. (2020). Genetic evidence for estrogenicity of bisphenol A in zebrafish gonadal differentiation and its signalling mechanism. *J. Hazard Mater.* **386**, 121886. doi:10.1016/j.jhazmat.2019.121886

Takatsu, K., Miyaoku, K., Roy, S. R., Murono, Y., Sago, T., Itagaki, H., Nakamura, M. and Tokumoto, T. (2013). Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. *Sci. Rep.* **3**, 3400. doi:10.1038/srep03400

Todd, E. V., Ortega-Recalde, O., Liu, H., Lamm, M. S., Rutherford, K. M., Cross, H., Black, M. A., Kardailsky, O., Marshall Graves, J. A., Hore, T. A. et al. (2019). Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. *Sci. Adv.* **5**, eaaw7006. doi:10.1126/sciadv.aaw7006

Uchida, D., Yamashita, M., Kitano, T. and Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J. Exp. Biol.* **205**, 711-718.

Ucuncu, S. I. and Cakici, O. (2009). Atresia and apoptosis in preovulatory follicles in the ovary of Danio rerio (Zebrafish). *Turk. J. Fish. Aquat. Sci.* **9**, 215-221. doi:10.4194/trjfas.2009.0214

Wang, Y. and Ge, W. (2004). Developmental profiles of activin βA, βB, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle1. *Biol. Reprod.* **71**, 2056-2064. doi:10.1095/biolreprod.104.032649

Wang, X. G., Bartfai, R., Sleptsova-Freidrich, I. and Orban, L. (2007). The timing and extent of 'juvenile ovary' phase are highly variable during zebrafish testis differentiation. *J. Fish Biol.* **70**, 33-44. doi:10.1111/j.1095-8649.2007.01363.x

Webster, K. A., Schach, U., Ordaz, A., Steinfeld, J. S., Draper, B. W. and Siegfried, K. R. (2017). Dmrt1 is necessary for male sexual development in zebrafish. *Dev. Biol.* **422**, 33-46. doi:10.1016/j.ydbio.2016.12.008

Yin, Y., Tang, H., Liu, Y., Chen, Y., Li, G., Liu, X. and Lin, H. (2017). Targeted disruption of aromatase reveals dual functions of cyp19a1a during sex differentiation in zebrafish. *Endocrinology* **158**, 3030-3041. doi:10.1210/en.2016-1865

Zhang, Z., Lau, S.-W., Zhang, L. and Ge, W. (2015). Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcgr) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. *Endocrinology* **156**, 3747-3762. doi:10.1210/en.2015-1039

Zhang, Z., Zhu, B. and Ge, W. (2015). Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Mol. Endocrinol.* **29**, 76-98. doi:10.1210/me.2014-1256

Zhang, X., Li, M., Ma, H., Liu, X., Shi, H., Li, M. and Wang, D. (2017). Mutation of foxl2 or cyp19a1a results in female to male sex reversal in XX Nile tilapia. *Endocrinology* **158**, 2634-2647. doi:10.1210/en.2017-00127

Zhu, B., Pardeshi, L., Chen, Y. and Ge, W. (2018). Transcriptomic analysis for differentially expressed genes in ovarian follicle activation in the zebrafish. *Front. Endocrinol. (Lausanne)* **9**, 593. doi:10.3389/fendo.2018.00593