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Zebrafish as an emerging model to study gonad development

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ABSTRACT

The zebrafish (*Danio rerio*) has emerged as a popular model organism in developmental biology and pharmacogenetics due to its attribute of pathway conservation. Coupled with the availability of robust genetic and transgenic tools, transparent embryos and rapid larval development, studies of zebrafish allow detailed cellular analysis of many dynamic processes. In recent decades, the cellular and molecular mechanisms involved in the process of gonad development have been the subject of intense research using zebrafish models. In this mini-review, we give a brief overview of these studies, and highlight the essential genes involved in sex determination and gonad development in zebrafish.

Contents

1. Introduction

The existence of two differentiated sexes is common among animals, and is critically important for sexual reproduction and the survival of a species. However, the mechanisms of sex determination are amazingly variable and poorly understood. In contrast to most other highly conserved developmental processes, no single gene network controls sex determination in all species. Consequently, a series of current studies have been working on this important open question: how is the highly diverse system suited to achieving reproductive fitness and success [1]? Zebrafish are increasingly popular as research organisms, especially in studies of embryonic development and as disease models [2,3]. In the extensive literature on embryonic development and biology of zebrafish, some attention has been given to their cellular reproductive processes. Recent progress in Cas9-related transgenic technology and single cell RNA-seq analysis also provides the opportunity to understand the cellular events of sex determination and gonad development in specific fish [4,5]. In turn, these data allow us to compare gonad development in zebrafish with that in other animals. Here we summarize current knowledge of the gene networks involved in sex determination and gonad development in zebrafish.

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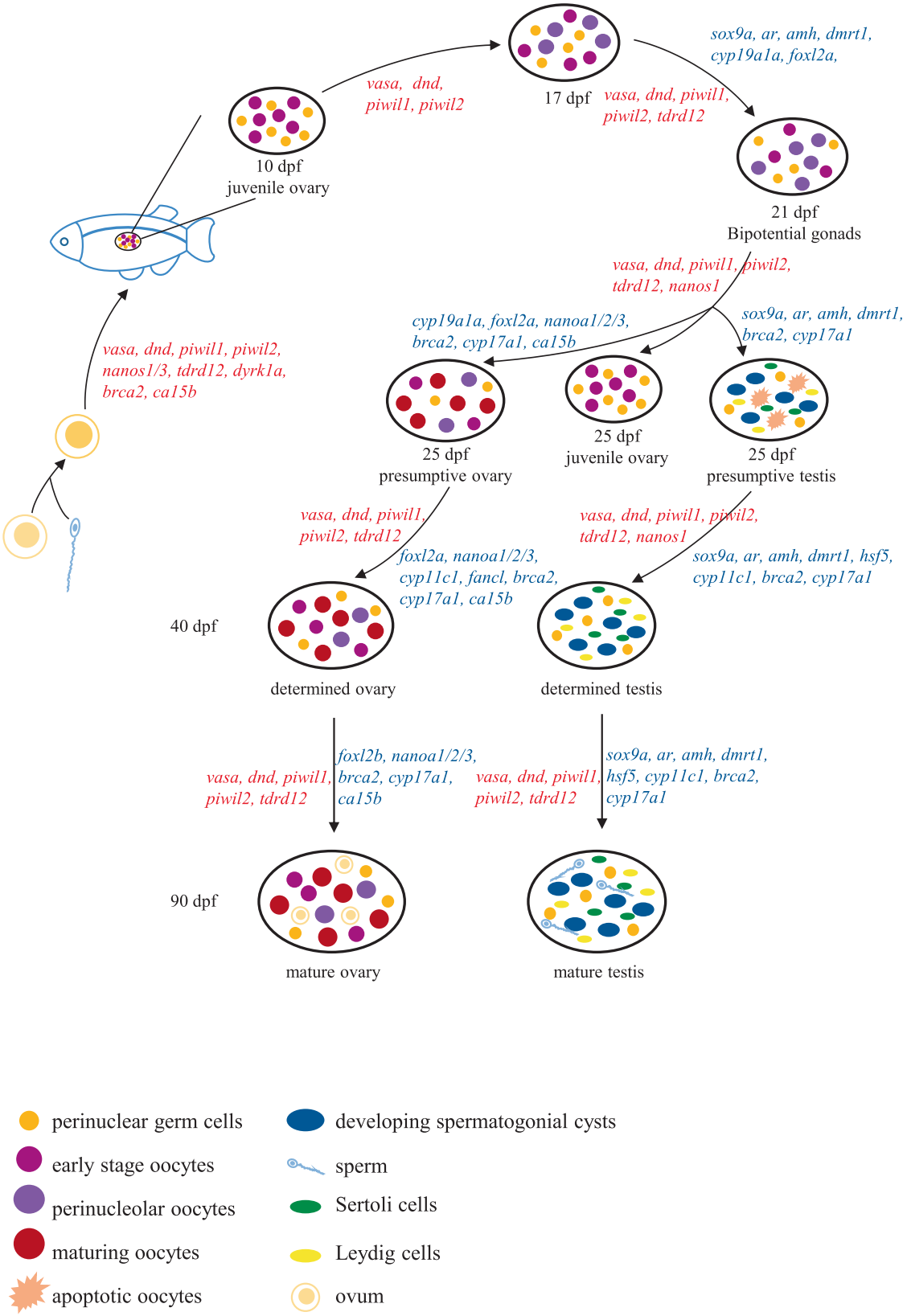

Fig. 1. Gene networks during gonad development in zebrafish. Different genes play important roles in different stages to complete the complex life process of sex determination and gonad development. Graphics with different colors or shapes represent different types of cells.

2. Sex determination and gonad development in zebrafish

In vertebrates, the male and female reproductive tracts are derived from the same embryonic tissue [6]. In other words, the gonad begins as a bipotential tissue. Sex determination refers to the key events involved in the differentiation of gonads into testis or ovary (Fig. 1). In zebrafish, the development of gonads first passes through an ovary-like stage. Early oocytes can be found in all gonads at 10 days post fertilization (dpf) [7,8]. While no signs of mitotic activity are typically recorded until 15 dpf [9], early diplotene oocytes can be observed with a clear juvenile ovarian structure [7,10], suggesting early vertebrate oogenesis. Selman et al. described the oogenesis process in zebrafish as having five stages, defined according to their morphology and karyotype in the ovary [11,12]. Based on this, sex differentiation is marked by the appearance of perinuclear oocytes, which occurs as early as 17 dpf [13]. The two patterns of differentiation appear in the gonads by 21 dpf, and meiosis is initiated in some gonads at 22 dpf [7]. Depending on multiple signaling networks, the juvenile ovaries maintain the oogenic pathway in about half of the individuals; the other half undergo apoptosis to initiate the "juvenile ovary to testis" gonad transformation process, ultimately transforming into testes [7,8,14]. By 25 dpf, gonads are still bipotential [7]. Three morphological states of gonads, including juvenile ovary with perinucleolar oocytes, presumptive ovary with maturing oocytes, and presumptive testis with apoptotic oocytes and developing spermatogonial cysts, can be observed by 31 dpf [15]. At 40 dpf, female gonads contain germ cells at various stages of oogenesis, whereas in other gonads, degenerative oocytes are observed and considered the first indication of spermatogenic activity [7,15].

3. Gene networks for reproductive development in zebrafish

The sex determination mechanism can be mainly divided into two types, genetic sex determination (GSD) and environmental sex determination (ESD). The GSD can either have a monogenic or a polygenic basis. XX/XY system and ZZ/ZW system are two typical systems of GSD. There are various sex determination mechanisms in fish, including GSD, ESD or GSD-ESD interactions. Zebrafish do not have the so-called "heteromorphic sex chromosomes" that determine sex in humans and several other species. Zebrafish displays polygenic sex determination and also affected by the environment. Laboratory strains lack a clear sex-linked locus of sex chromosomes, but wild strains from India have locus on Chr4 that acts as a ZZ/ZW sex chromosome system with environmental influence [16]. Researchers have known that environmental factors, such as water temperature for example, can alter the sex ratio in zebrafish populations [17]. But the gene networks involved in sex determination have remained unclear and sometimes controversial. Here we summarize the genes critical for sex determination and gonad development in zebrafish (Table 1).

3.1. Germ cell-specific genes

Correct specification and maintenance of germ cells is necessary for sexual reproduction in multicellular organisms. While germ cell specification varies among different species, the molecular factors are primarily conserved [18].

vasa and *dnd1* (previous name: *dnd*) are always used as germ cells specific markers. Maternally supplied *vasa* and *dnd1* are detected during the cleavage stage. It has been shown that the expression of these two genes is critical for germ cell migration, survival and maintenance [19–21]. Piwi genes have been identified as regulatory proteins responsible for stem cell and germ cell differentiation. In zebrafish, Piwi homologs, *piwil1* and *piwil2* are expressed in both male and female gonads. *piwil1* is expressed in primary germ cells in the embryonic genital ridge and adult gonads, where it is co-localized with *vasa*. Houwing et al. reported that the absence of *piwil1* triggered germ cell apoptosis during larval development. In these adult fish with reduced *piwil1* function, germ cells were maintained but displayed abnormal levels of apoptosis. *piwil2* is expressed in primordial germ cells (PGCs) from 3 dpf and found in the gonads of both adult males and females [22,23]. As with *piwil1*, the loss of *piwil2* also has marked effects, resulting in the failure of germ cells to differentiate into mature oocytes or sperm [24]. *nanos1*, which encodes a maternal mRNA, is expressed in PGCs during an early embryonic developmental stage, and is essential for the survival and migration of PGCs [25]. Tudor domain-related proteins (Tdrds), functioning as Piwi-interacting proteins, have been demonstrated to be involved in spermatogenesis. Dai et al. showed that *tdrd12*-deficient fish displayed reduced numbers of germ cells and germ cells eventually lost by 35 dpf. Furthermore, meiosis defects were also observed in *tdrd12* mutant-derived germ cells [26].

The correct expression of these genes is essential for healthy formation of PGCs and normal development of gonads. Homozygotic knockout zebrafishes or mutants of these germ cell-specific genes (*vasa*, *dnd1*, *piwil1*, *piwil2*, *nanos1* and *tdrd12*) are usually exclusively male with threadlike sterile testes; while these fish can mate with females and stimulate spawning, the eggs are unfertilized [21,23,26–28].

In addition, *ca15b* is expressed in PGCs in early embryos, whose expression is similar to *vasa*, suggesting *ca15b* is required for PGCs development [29] Besides, *dyrk1a* expression begins in the 2-cell phase and exists in all cells at the blastocyst stage in zebrafish, which is in turn related to the normal formation of PGCs. Overexpression of *dyrk1a* leads to decreased expression of two important factors related to the development of PGCs, *ca15b* and *piwil1*, resulting in decreased number of PGCs and disordered migration. Zebrafish *dyrk1a* is highly conserved with human *DYRK1A*, and this work may provide a possible mechanism for germ cell defects in Down syndrome patients [30].

3.2. Testis-associated genes

The correct differentiation and development of the testes is a fundamental prerequisite for reproduction. The genes, *sox9a*, *dmrt1*, *amh*, *ar* and *hsf5* play important roles in the "ovary to testis" stage and later testicular maturation stage in zebrafish, all of which are essential for proper differentiation and development of testes [31–34].

The expression of *SRY-box transcription factor 9a* (*sox9a*) in zebrafish reaches its first high peak at 18 dpf; the next peak is consistent with the *ar* expression peak at 22 dpf, when the bipotential gonads initiate differentiation to ovary or testis [35]. *sox9a* has been shown to be an upstream positive regulator of *amh* and an upstream negative regulator of *cyp19a1a* in the testes [36]. Webster et al. demonstrated that *doublesex and mab-3 related transcription factor 1* (*dmrt1*) is indispensable for the development of the testis but unnecessary for ovary development; *dmrt1* also facilitates male development through transcriptional regulation of the *amh* and *foxl2* genes [37]. *Anti-Müllerian hormone* (*amh*) is known to be a crucial factor for male gonad development. It is important for regression of Müllerian ducts, and for controlling the balance between proliferation and differentiation of germ cells in males [38]. The androgen receptors (*ar*) interact with androgens to exert functions in male development. The transcripts of *ar* peak at 16 dpf and 22 dpf, and are subsequently maintained at a high stable level across the presumptive male development stage, suggesting that it is essential for male differentiation and maintenance [35,39]. Saju et al. found that *heat shock transcription factor family member 5*

Table 1
Summary of genes required for gonad development.

Gene	Zebrafish	Mouse	Human
vasa	Important for germ line specification, survival, migration and maintenance, required for fertility in adult zebrafish [21]	Required for male but not essential for female during germ cell development, crucial for premeiotic differentiation in spermatogenesis [63]	Expressed in germ line, necessary for germ cell maintenance [64]
dnd1	Required for PGC migration and survival [20]	Expressed in PGCs, *Dnd1*-knockouts lead to germ-cell-free, sterile gonads [65]	Has been identified ESTs and genomic sequences encoding closely related genes in human [20]
piwil1	Essential for germ line maintenance, unessential for germ cell specification and early maintenance [23]	Essential for spermatogenesis [66]	Expressed in spermatocytes and spermatids [67]
piwil2	Required for germ cell differentiation and meiosis [24]	Much more predominant in female germ cells than in males [68]	Expressed in testis or embryonic cells, also important for the pathological process of various malignant tumors [69]
nanos1	Essential for PGCs survival, indispensable for maintaining the oocyte production [27]	Not concerned with germ cells development, expressed in maturating spermatids and oocytes [70]	Important for mRNA translation within chromatoid body, represses apoptosis in human germ cells [71,72]
tdrd12	Important for germ cell development and maintenance [26]	Important for spermatogenesis [73,74]	Involved in spermatogenesis [75]
ca15b	Required for PGCs development in early embryos and perhaps has an important role in oogenesis [29]	No detailed information	No detailed information
dyrk1a	Overexpression will alters the expression of some important factors (e.g. piwii1), leading to dysplasia of PGCs [30]	Involved in the migration and maintenance of PGCs [76]	Associated with Down Syndrome, related to cell proliferation and has other multiple functions [77]
sox9a	Important for male testis determination [31,35]	Reduced expression in the ovary and growing expression in the testis, heterozygous mutations do not lead to XY sex reversal [78–80]	Heterozygous *SOX9* mutations cause partial or complete XY sex reversal in the context of the skeletal malformation syndrome campomelic dysplasia [81,82]
dmrt1	Unessential for ovary development, indispensable for testis development [37]	Required for maintaining spermatogonial stem cells (SSCs) during steady state spermatogenesis, important for recovery of spermatogenesis after germ cell depletion [83]	Required for human testis differentiation, dmrt1 deficiency is related to focal testicular dysgenesis and sex-reversal [84,85]
amh	Important for regression of Müllerian ducts, controls the balance between proliferation and differentiation of germ cells in males [38]	Involved in Sertoli cell development, facilitate the expression of regulating factors in spermatogenesis [86]	Impacts a variety of fundamental processes within the ovaries and testes [87]
ar	Interact with androgens, essential for male development and maintenance, key to spermatogenesis and maintenance of ovarian function [41]	Essential for male reproductive development and spermatogenesis [88]	Crucial for spermatogenesis, *AR* mutations lead to disorders in male reproductive and developmental [59]
hsf5	Essential for proper spermatogenesis and fertility in males [40]	Essential for spermatogenesis [89]	Expression of *HSF5* protein is restricted to spermatocytes and round spermatids [90]
cyp19a1a	Encodes an aromatase limiting the rate of transformation from testosterone to estrogen,plays duple roles during sex differentiation in zebrafish [43]	No detailed information	Irregular expression related to the development of polycystic ovary syndrome (PCOS) [91]
foxl2	Crucial for ovary development and maintenance, *foxl2a* and *foxl2b* make a cooperation to conduct ovary development and maintenance [45]	Indispensable for follicular development and female fertility maintenance, continuous expression important for "ovarian somatic cells to testicular cells" transformation [92]	Required for granulosa cell development, mutations lead to granulosa cell tumors (GCTs) [93]
nanos2	A marker for germline stem cell, expressed in both ovarian and testicular pre-meiotic germ cells [47]	Expressed only in male gonocytes, inhibits meiosis and promotes male-type differentiation [94]	Testis-specific, expressed in prenatal germ cells and late stages of spermatogenesis [95]
nanos3	Only found in oocytes, *nanos3* mutants perform loss of <20um germ cells in juvenile ovary [47]	Important for germ cell development [96]	Expressed in embryonic stem cells, essential for maintaining normal germ cell numbers [97]
brca2	Critical for ovarian development [51,52], required for the development of embryonic kidney podocytes [53,54]	Limited information is available due to most homozygous *Brca2* mouse mutants display severe embryonic lethal phenotypes [60]	Involved in FA, breast and ovarian cancer [98]
fancl	Important for ovarian differentiation and development [46]	Necessary for germ cell proliferation maturation of oocytes, but not for the proliferation or maturation of spermatogonia in adulthood [46,58]	Involved in FA [99,100]
cyp17a1	Required for ovarian differentiation and maintaining male-typical SSCs and mating behaviors [55]	Deletion of *Cyp17a1* leads to infertility and sexual behavior defects due to the insufficiency of androgen [101]	Essential for the production of androgens and glucocorticoids, involved in prostate cancer [102]
cyp11c1	Necessary for oocytes maturation, testicular development and spermatogenesis [56,57]	Involved in congenital adrenal hyperplasia [103]	Involved in congenital adrenal hyperplasia and abnormalities in gonad [104,105]

(*hsf5*), whose expression increased from 35dpf, plays an important role in early stage of spermatogenesis and fertility in males [40].

In summary, homozygotic knockout zebrafishes or mutants of these testis-associated genes (*dmrt1*, *amh* and *ar*) usually display a female-biased sex ratio [37,38,41]. In particular, abnormal expression of *sox9a* can block the ovary-testis transformation in zebrafish juvenile. Male gonads lacking *dmrt1* expression lead to impaired *amh* gene expression, with the result that the expression of the *foxl2* gene is no longer restrained. Decreased oocyte apoptosis and intersex gonad development is observed with testicular dysgenesis and failed spermatogenesis. In contrast, female ovary development is normal in these *dmrt1* mutants [37]. Hypertrophic testes with more germ cells and fewer spermatozoa are observed in homozygous *amh* mutated males, on account of uncontrollable proliferation and defective differentiation of the germ line [38]. *ar*-null male zebrafishes appear with female secondary sex charac-

teristics and are sterile, having smaller testes and blocked spermatogenesis [41]. *hsf5*−/− mutant males are infertile with decreased sperm count, abnormal sperm morphology and decreased sperm motility, however, females are fertile [40].

3.3. Ovary-related genes

The ovaries, producing ova and secreting estrogen, are the core of the female gonads. Genes such as *cyp19a1a*, *foxl2*, *fancl*, *ca15b*, *nanos1*, *nanos2*, and *nanos3* jointly regulate the proper formation and function of the ovaries.

Low expression or no expression of *sox9a* prevent bipotential gonads from developing into male gonads; following this, gonads initiate differentiation to females with increased expression of *cyp19a1a*. The gene *cyp19a1a* encodes an aromatase that limits the rate of transformation from testosterone to estrogen, and plays dual roles during sex differentiation: low expression of *cyp19a1a* is needed for testis differentiation, while high expression of *cyp19a1a* is vital for ovarian differentiation and maintenance of female sex [42,43]. *foxl2* is crucial for ovary development and maintenance in mammals. There are two *foxl2* homologous genes in zebrafish [44], named *foxl2a* and *foxl2b*. The expression of *foxl2a* is higher than *foxl2b* during ovary differentiation and development, and the expression of *foxl2b* is more abundant in mature ovaries. The functions of *foxl2a* and *foxl2b* are complementary, and they cooperate to regulate ovary development and maintenance, with *foxl2b* playing a more specific role in ovary maintenance and preventing male transformation [45]. *fancl*, a member of the Fanconi/BRCA DNA repair pathway, is expressed in developing oocytes and spermatocytes. In particular, *fancl* is important for ovarian differentiation and development by progressing oocytes through meiosis, and meanwhile, maintaining *cyp19a1a* expression and down-regulating *amh* expression [46]. Besides, as we mentioned before, *ca15b* is expressed in PGCs in early embryos. Wang et al. also found that *ca15b* is expressed in oocytes in adult females, suggesting *ca15b* perhaps has an important role in oogenesis [29]. In addition, the expression of *nanos1* is required for maintaining oocyte production during female gonad development [27]. *nanos2* and *nanos3* are important for the maintenance of germline stem cells (GSCs) in the ovaries [47,48].

Abnormal expression of these ovary-related genes can each lead to respective abnormal phenotypes. The biosynthesis of estrogen is defective in *cyp19a1a*-null mutant zebrafishes, and although male *cyp19a1a* mutants are fertile, the effect on estrogen causes ovary-like gonads to disappear during sex differentiation and leads to all males having delayed male sex differentiation. In addition, dysregulated *cyp19a1a* expression gives rise to expression of other genes, such as *sox9a*, *amh*, *dmrt1* and *foxl2*, at abnormal stages during the gonad differentiation period [43]. *foxl2a* mutant homozygotes have a normal 1:1 sex ratio, while *foxl2b* disruption causes partial sex reversal in adult females. Both *foxl2a*−/− and *foxl2b*−/− mutants perform normal ovarian differentiation and oocyte development initially. However, after maturity, the testis-associated genes (*sox9a*, *amh* and *dmrt1*) are increasingly expressed, while in contrast, the ovary-related gene *cyp19a1a* is decreasingly expressed in *foxl2a*−/− or *foxl2b*−/− female mutants. Accelerated ovarian aging and abnormal meiosis of oocytes are detected in *foxl2a*−/− female adult zebrafishes, which is similar to conditions associated with premature ovarian failure (POF) in human patients [45,49]. In zebrafish, some of the *foxl2b*−/− homozygous female mutants undergo sex reversal from 180 dpf. Moreover, the *foxl2a* and *foxl2b* concurrently deficient females appear to undergo complete sex reversal [45]. *fancl* homozygous mutants develop as all fertile males due to female-to-male sex reversal. Oocytes are absent caused by abnormal apoptosis, and the expression of female-specific genes (e.g., *cyp19a1a*) is failed to maintaining and the expression of male-specific genes (e.g., *amh*) is upregulated, further leading to masculinized testes [46]. In addition, *nanos1* mutant females are completely sterile at 6 months post fertilization (mpf) [27], while *nanos3* mutant adult females are initially fertile, but transition into sterility after 5 mpf [47].

3.4. Other important genes

In addition to the genes described above, there are some genes that play important roles in the development of both male and female zebrafishes, regulating the development of their gonads. Here we introduce the functions of *brca2*, *cyp17a1* and *cyp11c1*.

Zebrafish *fancd1*(*brca2*) and *fancl* are homologous to human *FANCD1*(*BRCA2*) and *FANCL* and conserved in both structure and function, respectively [50]. They are two of Fanconi Anemia (FA) genes contributed to DNA repair and maintaining genome stability. The function of *fancl* has been described above. *brca2* is expressed both in the developing oocytes and in mature oocytes in adult zebrafish, however, in the testis, it is expressed in spermatogonia and developing spermatocytes, but not in mature sperm. *brca2* is required for establishing or maintaining oocyte nuclear architecture and critical for ovarian development. All homozygous *brca2* adult mutants are males due to oocytes failed to progress through meiosis at juvenile stage, resulting in female-to-male sex reversal. Adult males are infertile owing to abnormal meiosis of spermatocytes, subsequently leading to apoptosis of spermatocytes. Meanwhile, they are more susceptible to suffer testicular tumors [15,51,52]. And recently, Kroeger et al. and Drummond and Wingert found *brca2* is required for the development of zebrafish embryonic kidney podocytes in recent years respectively, which are given a new model to further study *BRCA2* functions in disease [53,54]. Besides, mutation of *tp53* can rescue the female-to-male sex reversal phenotype of both *brca2* and *fancl* mutants, but cannot rescue fertility in *brca2* mutants [46,51].

cyp17a1, participated in the steroidogenic pathway producing estrogens and androgens, is important for sexual trait development in zebrafish. Androgens is required for testicular development, maintaining male-typical secondary sex characters (SSCs) and normal mating behaviors. Meanwhile, estrogens are indispensable for ovarian differentiation. *cyp17a1*-deficient zebrafishes are all males due to the biosynthesis of estrogens is blocked. Simultaneously, males perform female-like characters, including light yellow anal fin coloration and dark black body pigmentation, and loss of parallel swimming and grasping leading to failed female spawning, which resulted from impairing androgen synthesis [55].

Zebrafish *cyp11c1*, encoded 11β-hydroxylase, is important for the biosynthesis of 11-ketotestosterone (11-KT) and cortisol. Zhang et al. found *cyp11c1* is expressed both in the testis and ovaries at 30 and 35 dpf, and then specifically expressed in the testis after 45 dpf. The sex ratio of *cyp11c1*−/− fishes has no significant difference compared to wildtype. Zhang et al. considered that endogenous cortisol synthesized by *cyp11c1* might be necessary for oocytes maturation, because exogenous cortisol treatment could partially rescue the defective mature processes of oocytes and decreased oviposition in females [56]. *cyp11c1*−/− males perform delayed and prolonged juvenile ovary-to-testis transition process [56], and show smaller testes, delayed spermatogenesis, reduced spermatogenesis efficiency and decreased spermatogenesis quantity, which can be rescued by 11-KT or testosterone treatment [57]. Meanwhile, the expression of *cyp17a1*, *amh* and *dmrt1* was significantly decreased during testis development of *cyp11c1*−/− males. In addition, functional gametes can be produced in both *cyp11c1*−/− males and females, but offsprings cannot be produced due to defective mating behavior [56].

4. Future prospects

Compared to asexual reproduction that cells simply create carbon copies of themselves, sexual reproduction allows the introduction of genetic diversity into a population. As an ancient feature of life on earth, it led to the impression that sex determination mechanisms are old and conserved. However, males and females are determined by diverse mechanisms, whereas proper activation essential for successful sex determination differs in various taxa. Indeed, a series of studies are working on these important open questions: What are the mechanisms determine sexes? What are the ways in which they manifest? Why so many ways of doing sex determination for one seemingly common result? What are the molecular players linking rapid turnover of sex determination in few systems?

The rapid development of genomics has allowed researchers to analyze this novel biological system at the molecular level. In addition, a full understanding of the diversity of gender determination mechanisms will require us to expand the breadth of study systems. As discussed, the process of sex determination in zebrafish undergoes great change after some genes are knocked out or mutated. In studies of mice, however, individuals with homologous gene knockouts did not show significant male or female sexual bias (such as *Fancl* mutant mice) [58], suggesting a significant difference in sex determination between zebrafish and mammals with a strong genetic sex-determining mechanism. In human populations, when we look at the correlation between these gene mutants and clinical cases, patients carrying these mutants often show issues with gonadogenesis, including hypogonadism and infertility. For example, *AR* mutations in human patients display androgen insensitivity syndrome (AIS), premature ovarian failure (POF) or other gonadal problems [59]. These data suggest that although there are differences in the mechanism of sex determination between zebrafishes and mammals, zebrafish still hold great promise as a model organism. In particular, zebrafish may be a good complementary model to study gene functions when homozygous lethality occurs in knockout mice. For example, most homozygous *Brca2* mouse mutants display severe embryonic lethal phenotypes [60]. Therefore, studies of gonad development in zebrafish can complement those of mammalian gonad development, and potentially provide entirely new ideas.

In addition, many groups have in recent years conducted Single-cell RNA-seq, an unbiased approach that has extended our understanding of heterogeneous tissues in embryonic studies [61,62]. There is no doubt that this technique will benefit studies of gonad development. The analysis of stage-specific gene expression profiles of individual gametogenic cells may provide unbiased and novel insights into their molecular details. Comparative and functional genomic data will allow researchers to figure out how new master sex-determination genes are incorporated into existing genetic networks and control sexual development. In this respect, zebrafish have many advantages as experimental animals. For instance, zebrafish provide relatively large fertilized eggs on a daily basis under simple laboratory conditions. In addition, the embryos and early larvae are transparent, allowing detailed cellular analysis of many dynamic processes.

Taken together, these advantages mean that future work with zebrafish aimed at understanding complex gonad development will likely support and improve our knowledge of physiopathology, with diagnostic as well as therapeutic potential in humans.

CRediT authorship contribution statement

Mengling Ye: Writing - original draft. **Ye Chen:** Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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