Review

Finding clues to the riddle of sex determination in zebrafish

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How sex is determined has been one of the most intriguing puzzles in biology since antiquity. Although a fundamental process in most metazoans, there seems to be myriad of ways in which sex can be determined – from genetic to environmental sex determination. This variation is limited mainly to upstream triggers with the core of sex determination pathway being conserved. Zebrafish has gained prominence as a vertebrate model system to study development and disease. However, very little is known about its primary sex determination mechanism. Here we review our current understanding of the sex determination in zebrafish. Zebrafish lack identifiable heteromorphic sex chromosomes and sex is determined by multiple genes, with some influence from the environment. Recently, chromosome 4 has been identified as sex chromosome along with few sex-linked loci on chromosomes 5 and 16. The identities of candidate sex-linked genes, however, have remained elusive. Sex in zebrafish is also influenced by the number of meiotic oocytes in the juvenile ovary, which appear to instruct retention of the ovarian fate. The mechanism and identity of this instructive signal remain unknown. We hypothesize that sex in zebrafish is a culmination of combinatorial effects of the genome, germ cells and the environment with inputs from epigenetic factors translating the biological meaning of this interaction.

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1. Introduction

In a little over three decades, zebrafish (*Danio rerio)* has gained eminence as a model organism to study diverse biological processes. This small tropical fish, native to India, made its humble beginning as a result of search for a vertebrate model system in which development can be studied in detail and also allow the use of genetic manipulation by Streisinger (Streisinger *et al.* 1981; Grunwald and Eisen 2002; Nüsslein-Volhard and Dahm 2002). These are relatively small and hardy fishes, have short generation times and can breed as frequently as weekly producing few hundreds of optically transparent embryos in each breeding. The zebrafish combines many advantages of other vertebrate and invertebrate models while bridging gaps between them and has become the model of choice for understanding diverse biomedical phenomenon. Due to its remarkable attributes, zebrafish has provided a wealth of information about vertebrate development, to our understanding of human disease and is poised to accelerate drug discovery (Nüsslein-Volhard and Dahm 2002). While many aspects of zebrafish biology have been well studied, our current understanding of zebrafish sex determination has largely been inadequate. Here we review our current knowledge of sex determination mechanisms in this model organism. We propose a potential role for epigenetic factors in determining the sex of the developing embryo in ectothermal vertebrates – like fishes – where sex determining modes are labile and how zebrafish could be useful model to study epigenetic modes and evolution of sex determination.

Keywords. Epigenetic factors; juvenile ovary; PGC; sex determination; zebrafish; 'sex-biased' epigenome

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2. Sex determination mechanisms – different means to same end

The mechanism by which sex is determined during development has fascinated biologists since antiquity. Given the importance of sexual reproduction in evolution, and since many metazoans are unisexual, it would appear that sex determination and differentiation are ancient and conserved. However, there exists a marked variation in primary sex determination mechanisms – the developmental program during which sex of the bipotential embryo is determined (Marın and Baker 1998; Williams and Carroll 2009). Broadly, sex could be determined either genetically or by environmental factors. In genetic sex determination whether an embryo develops as a male or female is governed largely by genes. In chromosomal sex determination, sex is determined by the presence of either cytologically distinct (heteromorphic) or similar (homomorphic) sex chromosomes. For example, in mammals the presence of a Y chromosome determines maleness. In *Drosophila*, sex is influenced by the ratio of sex chromosomes to sets of autosomes. In other cases of genetic sex determination, sex is determined by one or by many autosomal genes (polygenic mechanism) (table 1). In environmental sex determination, sex determination is under the control of environmental cues, generally acting at a critical window during development (table 1). Temperature (as in turtles), population density (mermithid nematodes), pH or even social cues like local sex ratio (many tropical fishes) are known to determine sex. In many instances environmental cues interact with the genotype to determine sex (Devlin and Nagahama 2002; Gamble and Zarkower 2012). While sex determination mechanisms have been well studied and conserved in mammals, and to some extent in birds, they are relatively poorly conserved and understood in other vertebrates.

3. Varied mechanisms of sex determination in fishes

Fishes are the most prevalent and diverse vertebrates with over 30000 species of teleost fishes (Nelson 2006). They also exhibit the most diverse means of sex determination among vertebrates with almost all the modes of sex determination (Devlin and Nagahama 2002; Desjardins and Fernald 2009). Some fishes like medaka (*Oryzias latipes*) have a XX-XY chromosome based sex determination similar to mammals, with the male determining master regulator genes on the Y chromosome. But this mode is not conserved even within the genus *Oryzias* (Matsuda *et al.* 2002). Other fishes have a ZW-ZZ based mechanism (Poecilia) or multiple sex chromosomes (Xiphophorus)(von Hofsten and Olsson 2005; Desjardins and Fernald 2009). In yet others, sex is determined by multiple genes. Such polygenic sex determination has been observed in fishes like zebra cichlid (*Metriaclima pyrsonotus)*(Ser *et al.* 2010). In many fishes sex determination and differentiation is influenced by a variety of environmental factors like pH, temperature, salinity, etc., along with social factors, many biochemical, developmental and endocrine signals (Baroiller *et al*. 2009). Of these, the effect of temperature has been best characterized with higher temperatures generally leading to male bias. Sex in fishes is also often sensitive to sex steroids (testosterone and oestrogen). Frequently environmental and social cues influence and override genetic component of sex determination and differentiation in fishes. Many fishes are hermaphrodites and others retain the ability to change sex in response to various environmental and social stimuli (Desjardins and Fernald 2009). Remarkably, even closely related species can have different mechanisms of sex determination indicating it's highly plastic and rapidly evolving nature. While the diversity of inputs that guide sexual fate in fishes is both baffling and intriguing, it also makes fishes attractive models to study evolution of sex determination.

4. Multiple genomic loci and environmental factors influence sex determination and differentiation in zebrafish

Notwithstanding numerous efforts, the primary mechanisms that determine zebrafish sex still remain controversial. Various reports suggest that sex differentiation in zebrafish is influenced by environment. Accordingly, multiple factors like nutrition, hypoxia, temperature, exposure to hormones and density have been implicated in influencing sex of the zebrafish (table 3). Numerous observations indicate that embryos that develop at higher temperatures during the critical period of gonadal transformation generally tend to develop as males (Abozaid *et al*. 2011, 2012). This is probably due to reduction or loss of oocytes, or reduction in the activity or levels of the aromatase, thereby, leading to male bias. Similarly, chemical inhibition of cytochrome p450 aromatase during this sensitive period also leads to elevated oocyte apoptosis and hence male bias (Uchida *et al*. 2004). Hypoxia also promotes male development possibly due to down regulation of several genes involved in the synthesis of sex hormones and a consequent increase in the testosterone/estradiol ratio (Shang *et al.* 2006). Although influenced by environment, zebrafish does not have a typical environmental sex determination like some turtles where temperature acts as a definitive cue (Ospina-Alvarez and Piferrer 2008).

Several observations suggest that sex is determined genetically in zebrafish (Liew and Orbán 2013). Selection experiments with specific crosses can yield predictable sex ratios often with a bias towards males. This suggests that sex is determined, at least in part, by genetic factors. Nevertheless, the lack of a single sex-linked master regulator gene so far indicates that sex is determined by complex

Table 1. Modes of sex determination and differentiation in vertebrates

Sex determination mode	group	Sex chromosome	Master/key gene	Epigenetic regulation
Genetic/chromosomal sex determination	Mammals	XX-XY	Sry	Yes X-inactivation
	Birds	ZW-ZZ	DMRT1	Yes Dosage compensation of Z-linked genes
	Lizards and snakes	XX-XY or ZW-ZZ	?	?
	Amphibians	XX-XY ZW-ZZ	DM-W (Xenopus)	?
	Medaka	XX-XY	DMY	?
	Xipophorus maculatus	XY/YY (male) XX/XW/YW (female)	?	?
	European Sea Bass	polygenic	?	Yes? *cyp19a1a* promoter methylation
	Swordtail (Platyfish)	WXZ	?	?
	Lake Victoria cichlid	B chromosome (female)	?	?
Environmental	Turtles (temperature)	?	?	?
	Amphibians (temperature)	?	?	?
	Fishes (temperature, social cues, pH etc.)	?	?	In few cases (European sea bass)

From Gamble and Zarkower (2012) and Devlin and Nagahama (2002).

Table 2. Comparison of developmental expression patterns of key sex differentiating factors

Protein	Zebrafish		Medaka		Mammals	
	Male	Female	Male	Female	Male	Female
Sox9	n/a	n/a	n/a	n/a	Testis (sertoli cells)	–
Sox9a	Testis (sertoli cells)	Juvenile ovary	Low levels in testis	Ovary	n/a	n/a
Sox9b	–	Ovary and oocytes	Testis	Low levels in ovary	n/a	n/a
DMRT1	High in testis, germ cells	Low in ovary, germ cells	?	?	Testis	–
Amh	Testis (sertoli cells)	Adult ovary	Testis (sertoli cells)	Ovary (follicular cells)	Yes (leydig and sertoli cells of testis)	Yes
Cyp19a1a (p450 aromatase)	–	Ovary (follicular cells)	–	Ovary (follicular cells)	?	?
FoxL2	–	Ovary	–	Ovary (follicular cells)	–	Ovary (granulosa cells)

From Siegfried (2010) and Cutting *et al*. (2013).

interaction of multiple genes. A recent study has analysed sex ratios of many families of zebrafish (Liew *et al.* 2012). It was observed that zebrafish exhibited large variations in the sex ratios between families. Furthermore, sex ratios of different batches of offspring from the same breeding pair were similar (Liew *et al.* 2012). This suggests that sex determining factors in zebrafish are heritable. The wide-ranging sex ratio across the families is typical of a complex trait influenced by multiple genes (table 3). Hence, zebrafish, like several other fishes (European sea bass) has a polygenic sex determination (Liew *et al.* 2012; Liew and Orbán 2013).

In polygenic sex determination, sex is a result of combinatorial action of multiple alleles on loci that are typically dispersed throughout the genome. Recently few attempts have been made to identify such allelic variations by genome wide analysis (Bradley *et al.* 2011; Anderson *et al.* 2012; Liew *et al.* 2012; Liew and Orbán 2013; Howe *et al.* 2013). Bradley *et al*. identified 2 sex linked loci in ABXIN cross (Bradley et al. 2011). One of the loci was on chromosome 5 encompassing the gene *dmrt1*. Two SNPs within *dmrt1* gene were suggested as functional candidates – a C/A polymorphism causing a non-synonymous D259E substitution and a A/G SNP at the cis-regulatory motif in the 3′ UTR of mRNA regulating its stability (Bradley *et al.* 2011). The sex-associated locus on chromosome 5 also contains *fancg* in addition to *dmrt1*. Whether *fancg* has any role in sex determination and differentiation needs further investigation. The other locus was on chromosome 16 including the gene *cyp21a2* that encodes a 21-hydroxylase involved in corticosteroid biosynthesis. It has been suggested that Cyp21a2 may play a role in ovary to testis transformation in protogynous groupers (Lee *et al.* 2002). 4 allelic variants leading to amino acid substitutions-G141R, V375M, Y380H and V527A- were predicted to be functional candidates (Bradley *et al.* 2011). A recent genome sequencing study also identified chromosome 16 as a sex-associated chromosome. However, the sex-linked loci identified on chromosome 16 in this study were different from the earlier study (Howe *et al.* 2013). Sex linked loci have also been identified on chromosome 4 and 3 (Anderson *et al.* 2012). While the chromosome 3 loci contains few candidate genes like *hsd17b1* that may play a role in sex determination, it was found associated with sex in only one of the families. On the other hand, the locus on chromosome 4 lacks any known candidate sex influencing/determining genes. Taken together, the lack of a master sex-linked loci or sex chromosome and identification of five different sex-associated loci from multiple independent genome-wide studies, even for a strong candidate gene like *dmrt1*, suggest that sex in zebrafish is a complex trait influenced by multiple genes. These observations also suggest that genetic factors influencing sex may differ among different strains and/or environmental conditions in zebrafish (Bradley *et al.* 2011; Anderson *et al.* 2012; Howe *et al*. 2013; Liew and Orbán 2013). Such strain dependent sex determination mechanisms exists in other fishes, for example, in guppies (*Poecilia*

Table 3. Factors affecting sex determination and differentiation in zebrafish

Factor involved in sex determination	Observation	Remarks	References
Temperature	Elevated temperature-male biased sex ratio	Embryos grown at 35C during thermosenstive period of gonadal differentiation	Abozaid *et al*. 2011, 2012
Hypoxia	Increased hypoxia-male		Shang *et al.* 2006
Sex steroids	Aromatase inhibitors-male bias	Affects Cyp19a1a levels	Uchida *et al.* 2004
Nutrition	Well nourished/fed- female bias		Lawrence *et al.* 2008
Density	Low density –female development		Nüsslein-Volhard and Dahm 2002
Genetic	Heritable but variable sex ratios	No heteromorphic sex chromosomes Polygeneic (broad range of sex ratios) Chromosome 4 sex associated? Female heterogamy (ZW-ZZ)	Liew et al. 2012

reticulata)(Volff and Schartl 2001). This points to a highly plastic and rapidly evolving genetic factors in zebrafish sex determination.

5. Is chromosome 4 a sex chromosome?

Many studies have shown that zebrafish has chromosomes of similar size and morphology, leading to uncertainty about existence of sex chromosomes (Daga *et al.* 1996; Gornung *et al.* 1997; Amores and Postlethwait 1999). Analysis of meiotic chromosomes and synaptonemal complex karyotyping of zebrafish has revealed that unlike medaka, zebrafish lack sex chromosomes (Sola and Gornung 2001; Traut and Winking 2001; Wallace and Wallace 2003; Liew and Orbán 2013). However, a study that analysed the karyotype of zebrafish sourced from wild in India indicated that zebrafish females are heterogametic sex (Sharma *et al.* 1998). A recent genome-wide study based on the population genomic analysis of RAD-tags to identify sex-linked SNPs indeed suggests that zebrafish in nature have ZW-ZZ sex determination mechanism with females being heterogametic (ZW)(Wilson *et al.* 2014). This study found that for a given allelic variant all individuals homozygous for one allele (e.g. *m/m* on Z chromosome) developed as males, most heterozygotes (*m/f*) developed as females representing ZW karyotype and the rare occurrence of a homozygous *f/f* genotypic female was due to mating of a sex reversed phenotypic male (ZW) with a genotypic female (ZW). It was also observed that although some fish with putative female genotype (*f/m*) developed as males, no genotypic males (*m/m*) developed as a female. These observations suggest that the *f* allele is necessary, but not sufficient, to determine female fate or this sex-linked locus is dosage sensitive with two doses ensuring male development while one copy favours, but does not assure, female development. The female-to-male sex reversal observed could be due to the influence of environmental factors (nutrition, density etc.) and genome (and their effects on germ cell numbers) favouring male pathway (Wilson *et al.* 2014).

The sex-linked SNPs are found clustered at the tip of right arm of chromosome 4. Interestingly, earlier studies had also showed that this region of chromosome 4 harbours a major sex determining region indicating that it could be sex chromosome. Several observations suggest that chromosome 4 could be an ideal candidate sex-chromosome in zebrafish. This region of chromosome 4 is poor in protein coding genes, is enriched in 5S-rRNA genes, satellite repeats, has high GC content, and is mostly heterochromatic and late replicating with low recombination rates. These distinctive features of the Q arm suggest that it could be remnant of, may be or might be evolving into a sex chromosome in the population (Howe *et al*. 2013). However, this locus on chromosome 4 lacks any known candidate sex influencing genes. An interesting candidate could be miRNA (mir430) that regulates genes involved in PGC and its migration (Mishima *et al.* 2006; Anderson *et al.* 2012).

Intriguingly, the laboratory strains of zebrafish (AB and TU) seem to lack or have greatly weakened sex determinants on this locus of chromosome 4. Since these strains can make males and females despite the lack of full complement of natural sex determinants, it suggests the existence of a functional sex determining mechanism. This could be due to unmasking of 'weak sex determinants' of the polygenic system and/or unmasking of the latent and pre-existing environmental sex determination mechanisms (Wilson *et al*. 2014). The identification of sex-associated loci on other chromosomes (3, 5 and 16) may represent either unmasking of the weak determinants or rapid evolution of new sex determination modes. In addition, the teleost-specific genome duplication and the possible role of epigenetic factors in sex determination and differentiation pathways (see later)

may also explain, at least partly, the plasticity in their sex determination modes

6. Molecular basis of sex differentiation in zebrafish

Among vertebrates, sex determination in mammals has been well studied and understood. In mammals, the presence of a Y-linked master regulator *SRY* – determines male development. *Sry* is a HMG-box transcription factor which regulates the expression of *Sox9* and other downstream regulators (like DMRT1) to initiate male development cascade (Kashimada and Koopman 2010; Wilhelm *et al.* 2013). Sox9 is one of the most conserved regulators of male development across vertebrates. In mammalian testicular development *Sox9* activates *Fgf9* expression which reinforces Sox9 and Fgf9 expression. Sox9 also activates Anti-Müllerian hormone (Amh) which mediates degeneration of Mullerian ducts (Cutting *et al.* 2013). In the absence of, female developmental pathway is initiated by players like R-spondin/Wnt4/b-catenin and FoxL2 *Sry*.

Unlike other developmental programs, sex determination mechanisms are extremely diverse making identification of players challenging. For example, Sry, is absent outside of eutherian mammals and in many vertebrates the identities of such 'master switch' has largely remained elusive. Yet, remarkably, this vast diversity is almost entirely limited to upstream cues and factors that trigger sex determining pathway as signalling networks and genes downstream in the sex determination and differentiation pathway are conserved. Often a downstream gene in this cascade gains the role of 'master switch' and genes frequently get shuffled in hierarchy. Hence although *SRY* itself appears to be unique to mammals, many downstream genes known to play a role in mammalian sex determination and differentiation are also known to be expressed in a sex specific manner in other vertebrates, including fishes (Wilkins 1995; Morrish and Sinclair 2002; Herpin and Schartl 2008; Siegfried 2010). Owing to the whole genome duplication during their evolution, many of these genes are also duplicated in fishes.

The molecular mechanisms of sex differentiation – the developmental pathways of gonadal differentiation- in zebrafish are well understood. Though gonochoristic, zebrafish exhibits juvenile hermaphroditism with the early bipotential gonad forming immature 'juvenile ovaries' that further matures into adult ovaries in females or transforms into testes in males (Maack and Segner 2003; Wang *et al.* 2007b). Various studies have analysed the expression patterns of genes during gonad development of zebrafish (table 2) (von Hofsten and Olsson 2005; Jørgensen *et al.* 2008; Sreenivasan *et al.* 2008; Siegfried 2010). DMRT1 is the most conserved and characterized downstream component of sex determination and has been shown to be involved in sex determination and differentiation in a wide variety of phylogenetically divergent groups like corals, annelids, arthropods and vertebrates- from fishes to mammals (Matson and Zarkower 2012). DMRT1 is one of the key targets of the mammalian *Sry* along with *Sox9*, and like *Sry,* determines testis and male fate in almost all cases (Matson and Zarkower 2012). *dmrt1* localized on the Z chromosome in avians (Kopp 2012) and a duplicated copy of *dmrt1-dmrt1by* or *Dmy*- present on the neo Y chromosome in medaka acts as a master regulator of testis and male development similar to *Sry* in mammals (Matsuda *et al*. 2002). Many elements of the vertebrate sex determination pathway converge on activation or inhibition of *Sox9* (Morrish and Sinclair 2002; Munger and Capel 2012). Zebrafish has 2 paralogues of *sox9- sox9a &b,* that are expressed differentially with *sox9b* mainly restricted to oocytes and *sox9a* being expressed in other somatic tissues and also in gonads (Chiang *et al*. 2001). Although fishes lack Mullerian duct, AMH is a key player in promoting male development and acts as a male switch in Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori *et al.* 2012). Expression of *dmrt1, sox9a and amh* are restricted to or are higher in the developing male zebrafish gonad in accordance with their role in determining male fate(Chiang *et al.* 2001 ; Guo et al. 2005; Rodríguez-Marí et al. 2005 ; Siegfried and Nüsslein-Volhard 2008 ; Jørgensen *et al.* 2008; Siegfried 2010) (table 2).

A striking difference between mammalian and non-mammalian sex determination modes is the influence of oestrogen on ovarian differentiation pathways in the latter. Conversion of androgens to oestrogens is mediated by ccytochrome p450 aromatases like Cyp19a. In many fishes inhibition of aromatases or exposure to androgens can cause masculinization even in genetic females. Conversely, over-exposure to oestrogens can promote female developmental pathways. By regulating sex steroid ratios, *cyp19a* plays a key role in sex determination and differentiation in non-mammalian vertebrates, including fishes. Zebrafish has two copies of *cyp19a1-cyp19a1b* that is expressed mainly in brain and *cyp19a1a* that is expressed predominantly in ovaries (Trant *et al*. 2001). During the juvenile ovary stage of zebrafish development, Cyp19a1a expression is seen in the gonads of all fishes. With the histological maturation, Cyp19a1a expression becomes restricted to developing ovaries (Rodríguez-Marí *et al*. 2005; Siegfried and Nüsslein-Volhard 2008; Siegfried 2010) (table 2). FoxL2, a forkhead transcription factor, is perhaps one of the most conserved components of vertebrate ovary determining pathway. It plays a critical role in mammalian female sex pathway by promoting Cyp19 expression and suppresses testis fate by repressing Sox9 (Pannetier *et al*. 2006; Wang *et al.* 2007a ; Uhlenhaut et al. 2009). Generally, in gonads FoxL2 is co-expressed along with aromatases. The expression pattern of *foxL2* in zebrafish has not been well characterized. Like many other factors in zebrafish, *foxL2* is initially expressed in the juvenile ovary but later its expression is restricted to

presumptive ovaries and excluded from the testis, similar to mammals (Siegfried and Nüsslein-Volhard 2008; Siegfried 2010).Other factors like R-spondin and Wnt-4 are also likely to play a role in female sex determination as in many other vertebrates (Siegfried 2010).

Although many key players like DMRT1, Sox9a, Cyp19a1a, Amh show sexually dimorphic expression patterns in accordance with their expected role in sex determination (table 2), it is unclear which of these, if any, is the key trigger in zebrafish sex determination cascade. Since many of the players implicated in sex are downstream in the pathway, it could be possible that their expression pattern is the result of the sex of embryo determined by an as yet unknown trigger. The identity of such a trigger is not known at present in part due to the difficulty in assigning sex morphologically during the critical period of early sex development.

7. Role of PGC in sex determination in zebrafish

While sex influences somatic tissue, the role of primordial germ cells (PGCs) – that eventually give rise to sperm or ova- in this process remains an open question. In mammals, although germ line is not required for testis fate determination and morphogenesis, it is likely that germ line and somatic tissues cooperate in the regulation of female sex determination. However, how germ line affects sexual fate of the surrounding somatic tissue is not understood (McLaren 1991; Brennan and Capel 2004; Guigon and Magre 2006). Similarly in many teleosts, including zebrafish and medaka, PGCs have a dramatic effect on the fate of somatic tissues, especially ovary. In medaka, although sex is determined by XX-XY chromosome based mechanism, ablation of germ cells results in the development of XX males (Kurokawa *et al.* 2007). Similarly in zebrafish loss of PGCs by various means and in mutants in which oocytes undergo apoptosis, gonadal development is biased to testes fate and leads to development of males (Slanchev *et al*. 2005; Houwing *et al.* 2007; Siegfried and Nüsslein-Volhard 2008; Rodríguez-Marí *et al.* 2010; Pradhan *et al*. 2012). A closer histological examination of developing gonads in these fishes during early stages (~25 dpf) reveals the presence of juvenile ovary in both germ cells depleted and control fishes, albeit without germ cells in the mutants. At later stages of sexual differentiation (~40–50 dpf) while similar proportion of control fishes had ovary and testis, in all the germ cells depleted fishes the juvenile ovary develops into adult testis (Slanchev *et al*. 2005; Siegfried and Nüsslein-Volhard 2008). These studies suggest that the undifferentiated gonads of PGC deficient fishes initially develop normally as bipotential juvenile ovaries, with no apparent abnormalities, which later transdifferentiates into testis in all fishes. Comparison of sex-specific markers reveals that during the early stages (~19 dpf) expression of *cyp19a1a* and *amh* is similar to wild-type fishes but in PGC defective fishes as sex differentiation progresses female specific markers like *cyp19a1a* and *foxL2* are down regulated followed by induction of testis specific genes like *amh* (Siegfried and Nüsslein-Volhard 2008). It is likely that loss of germ cells alters the hormonal equilibrium through the steroidogenic pathway and tilts the developmental balance towards male fate. Germ cells appears to be necessary even in adult fishes to maintain ovarian fate as upon ablating germ line in adults, female fishes revert to phenotypic males (Dranow *et al.* 2013). Surprisingly, few of these sex reverted males are fertile indicating that even the germ cells switch sex in line with the somatic tissue (Dranow *et al.* 2013). A critical factor in this decision could possibly be the number of oocytes in juvenile ovary. It has been observed that juvenile ovaries show variable numbers of oocytes in wildtype fishes- those with fewer oocytes having a greater tendency to develop into males (Wang *et al*. 2007b). In many teleosts, an increase in the number of germ cells in presumptive females relative to males is one of the earliest signs of sexual dimorphism (Nakamura *et al.* 1998) and this could be key step in male sex determination. Interestingly, mutation in medaka Amh receptor gene results in germ line over proliferation and XY-sex reversal (Kurokawa *et al*. 2007). Whether the germline is predisposed to male or female fate before gonadal differentiation to actively determine sex in zebrafish is not known (Siegfried and Nüsslein-Volhard 2008).

Based on these results, it has been hypothesized that an oocyte derived 'signal' acts on the bipotential somatic gonad to induce a female-specific developmental program. If this 'signal' is above threshold ovarian development is reinforced, else testis development is initiated as a default program and oocytes undergo apoptosis. This oocyte derived signal may either induce precursor pre-follicular cells to adopt granulosa cell fate instead of Sertoli cell in juvenile ovary and/or inhibit testis development. A possible function of this proposed signalling molecule could be production of oestrogens. The identity of the 'signal' from the meiotic oocytes that 'instructs' the somatic tissue to commit to and maintain the ovarian fate has, however, been mysterious. In mammals several oocyte derived paracrine factors like TGFβ, BMP15, FGF8, GDF9, etc., are known to have role in granulosa and theca cell development and function (McLaren 1991; Gilchrist *et al.* 2004). A recent study suggests that FSH signalling could activate Sox9a/p53 pathway to mediate transdifferentiation of follicular cells to Sertoli cells during juvenile ovary transition (Sun *et al.* 2013). It is likely that these factors, along with others, regulate granulosa cell development in zebrafish probably by oestrogen biosynthesis and *cyp19a1a* expression to determine sex. This hypothesis may be a common theme in many teleosts since germs cells influence the fate of somatic sex differentiation in medaka, trout and others. The mechanisms by which germ line influences somatic sex may thus share some similarities between fishes and mammals.

8. Epigenetic mechanisms and sex determination

Cell fate decisions during development are often 'remembered' and 'inherited' through successive cell divisions by means of epigenetic processes- heritable changes in the expression patterns that are not due to DNA sequence changes (Wu *et al*. 2009). Epigenetic regulation is generally due to covalent modifications of histones (mehtylation, acetylation, etc.), DNA (methylation of cytosine residues) along with non-coding RNA (Piferrer 2013). As each new lineage forms within a developing metazoan embryo, epigenetic processes sets up a unique pattern of expressed and repressed genes derived from the same genetic code. Hence it has been hypothesized that the evolution of epigenetic machinery was a necessity for multicellularity (Jablonka and Lamb 1998).

Epigenetic regulation plays a major role in dosage compensation in both vertebrates and invertebrates (Meller *et al*. 1997; Teranishi *et al.* 2001; Bisoni *et al*. 2005; Roeszler *et al.* 2012; Piferrer 2013). The role of epigenetic modifications in germ line and sex differentiation has also been well known, especially in mammals (Piferrer 2013). Loss/mutation of key epigenetic regulators is accompanied by severe dysregulation of several genes involved in sex determination and differentiation (Katoh-Fukui *et al.* 1998, 2012; Biason-Lauber *et al*. 2009; Kuroki *et al*. 2013). Whether epigenetic mechanisms play a role in determining sex in non-mammalian vertebrates is largely unclear.

The role of epigenetic modifications during differentiation of various somatic lineages is well documented, particularly in mammals. Vertebrate gonads, unlike other tissues, are bipotential at early stages of development and can commit to either testis or ovary fates in response to several cue viz., genetic, endocrine and environmental. These cues determine sexual fate by activating either the testis or ovary fate and by repressing the other pathway resulting in sexually dimorphic expression of genes. It is likely that epigenetic factors play a role in establishing and maintaining stable and spatiotemporally restricted expression of sex-determining and influencing genes by integrating a host of environmental and genetic cues. Accumulating body of evidence suggests that epigenetic mechanisms indeed play a role in non-mammalian vertebrate sex determination and differentiation, particularly in species where sex is influenced by environment. Sex steroid ratios play an important role in determining and influencing sex in most non-mammalian vertebrates. A prime target of upstream cues for modulating steroid levels is the aromatase Cyp19a1. In European sea bass (*Dicentrarchus labrax*), sex is determined by a polygenic system but is also equally influenced by temperature. The juvenile males have lower expression of Cyp19a1a compared to females. This difference in expression levels is due to significantly higher levels of DNA methylation in the *cyp19a1a* promoter in presumptive gonadal tissue, but not in brain. A positive correlation was observed between increasing temperature and methylation. An increase in *cyp19a1a* methylation in response to higher temperature and consequent lower expression of *cyp19a1a* results in masculinisation. Functional analysis of the promoter indicated that DNA methylation suppresses the ability of SF1 and FoxL2 to induce transcription of *cyp19a1* (Navarro-Martín *et al.* 2011). Similarly in Japanese black porgy (*Acanthopagrus schlegeli)*- a sequential protandrous hermaphrodite, *cyp19a1* promoter is hyper-methylated in the inactive ovarian tissue (Piferrer 2013). In addition, in alligators, male promoting temperature results in promoter hypermethylation of *cyp19a1* while female promoting temperature results in hypermethylation of *sox9* locus (Parrott *et al*. 2014). In red eared slider turtles where sex is determined by temperature, the promoter region of *cyp19a* in the vicinity of FoxL2 and SF1 binding sites are hypomethylated at female promoting temperature while a shift from male- to female promoting temperature results in reduction in DNA methylation at this locus (Matsumoto *et al.* 2013). These observations strongly suggest that the epigenetic regulation of *cyp19a1* could be one of the 'universal' themes in determining sex in non-mammalian vertebrates (Piferrer 2013). Whether epigenetic regulation of cyp19a1 also governs sex determination and differentiation in zebrafish, especially given the prevalence of sex reversal of genotypic males to females, is not known. Its attributes make zebrafish an ideal model system to understand the role of epigenetic players in sex determination and differentiation pathways and how environmental factors (and other physiological cues) affect this process.

9. Conclusions and perspectives

Despite numerous efforts, our understanding of the mechanisms by which sex is determined in zebrafish is incomplete. So far, observations from several studies suggest that sex in zebrafish is determined by multiple genes (reviewed in Liew and Orbán 2013), is influenced by primordial germ cells and often also by environmental factors like temperature. A recent study identified the right arm of chromosome 4 as a sex chromosome in natural strains of zebrafish with a ZW-ZZ type sex determination.

However, many important questions remain unanswered. The identity of the molecular players involved in establishing zebrafish sex still remain elusive (Wilson *et al*. 2014) although downstream signalling cascades involved in sex determination and differentiation in zebrafish are conserved with other vertebrates. Although sex-lined region has been recently identified on chromosome 4 in natural strains of zebrafish, this region lacks any protein coding genes. Elucidating how this region of chromosome 4 determines sex and the identity of the upstream candidate gene/s that triggers the sex determination and differentiation cascade requires immediate attention to understand the molecular basis of sex determination in zebrafish. Recent advances in next generation sequencing and other techniques

would aid in identifying new genes and their regulatory networks. An interesting candidate gene from chromosome 4 could be miRNA *mir-430* (Mishima *et al.* 2006; Anderson *et al.* 2012). Transcriptome and RNA-seq analysis in developing gonads and germ cells would provide insights into the possible role of such regulatory RNAs (miRNA, piRNA, lncRNA, etc.), splice variants and alternate transcripts in influencing sex. Such sexually dimorphic splicing has been observed for *vasa*(Krøvel and Olsen, 2004). Similarly, the role and the mechanisms by which germs cells determine and/or influence somatic sex and how environment modulates these outcomes also needs attention. What is the identity of the instructive 'signal' from developing oocytes which 'instructs' the somatic tissue to commit to ovarian fate? What is its mechanism of action? What are the triggers and 'modifiers' of this signal? With increasing evidence of epigenetic regulation of *cyp19a* expression in various vertebrates, the possibility of epigenetic regulators integrating and interpreting cues from genome, environment and germ cells to determine phenotypic sex in zebrafish needs to be explored.

We propose that sex in zebrafish is a result of complex interaction between genome, germ cells and environment along with the epigenetic factors playing a role in mediating these processes. Since sex in fishes is plastic and a threshold phenotype, the cumulative effect of genome along with other factors like environment on male and female promoting factors acting on somatic cells of gonads (for example, sex steroid ratios, factors affecting germ cell numbers) and PGCs (proliferation rate, survival) would contribute to sex determination in zebrafish. Understanding the role of epigenetic modifications in sex determination pathways can provide novel insights to solve the complex puzzle of sex determination in fishes. Seeking clues to the riddle of sex determination would not only further our understanding of the biology of zebrafish but can also provide insights into factors influencing evolution of sex determination modes and the effect of changing environment on sex ratios in fishes and reptiles.

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