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MINIREVIEW

Sex determination and sexual differentiation in the avian model

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The sex of birds is determined by the inheritance of sex chromosomes (ZZ male and ZW female). Genes carried on one or both of these sex chromosomes control sexual differentiation during embryonic life, producing testes in males (ZZ) and ovaries in females (ZW). This minireview summarizes our current understanding of avian sex determination and gonadal development. Most recently, it has been shown that sex is cell autonomous in birds. Evidence from gynandromorphic chickens (male on one side, female on the other) points to the likelihood that sex is determined directly in each cell of the body, independently of, or in addition to, hormonal signalling. Hence, sex-determining genes may operate not only in the gonads, to produce testes or ovaries, but also throughout cells of the body. In the chicken, as in other birds, the gonads develop into ovaries or testes during embryonic life, a process that must be triggered by sex-determining genes. This process involves the Z-linked *DMRT1* gene. If *DMRT1* gene activity is experimentally reduced, the gonads of male embryos (ZZ) are feminized, with ovarian-type structure, downregulation of male markers and activation of female markers. *DMRT1* is currently the best candidate gene thought to regulate gonadal sex differentiation. However, if sex is cell autonomous, DMRT1 cannot be the master regulator, as its expression is confined to the urogenital system. Female development in the avian model appears to be shared with mammals; both the FOXL2 and RSPO1/WNT4 pathways are implicated in ovarian differentiation.

Introduction

Sex determination can be defined as the earliest developmental event whereby sex is established. In birds and mammals, sex determination occurs at fertilization with the inheritance of the sex chromosomes. Sexual differentiation subsequently occurs and involves gonadal sex differentiation, producing either ovaries or testes. As a developmental pathway, sex determination must be a very ancient process, being linked to the sexual reproduction that has been a key driving force of evolution. The development of a sexual phenotype, usually male or female, generally occurs during embryonic development and is regulated by genetic and hormonal pathways. In vertebrates, sex can be determined by either environmental or genetic factors [1,2]. Genetic sex determination is observed in mammals and birds, with both groups having defined sex chromosomes. However, the ZZ/ZW sex chromosomes of birds are unrelated to the XX/XY sex chromosomes

Abbreviation
DMRT1, Double-sex and Mab-3 Related Transcription Factor #1.

of mammals, having evolved from a different pair of autosomes. Hence, birds lack *SRY*, the master testis-determining gene of therian mammals (marsupials and 'placentals') (Fig. 1A). This minireview focuses on recent advances in our understanding of avian sex determination and how this has informed our views of vertebrate sex determination in general. Unresolved questions associated with avian sex determination are also considered.

Evidence that sex determination is cell autonomous in birds

Most of our knowledge of avian sex determination comes from studies on the chicken (*Gallus gallus domesticus*), which has long been a key model for developmental biologists and for which complete genome sequence is now available [3]. The sex of chickens and other birds is determined genetically by the inheritance of sex chromosomes. Males have ZZ sex chromosomes and females have ZW sex chromosomes. The Z is large and carries the key candidate testis determinant, *DMRT1*. The smaller W has few bona fide genes and is largely heterochromatic (Fig. 1B). Genes carried on one or both of these sex chromosomes are thought to control gonadal differentiation during embryonic life, producing testes in males (ZZ) and ovaries in females (ZW). In the chicken, the initially bipotential gonads commence morphological differentiation into ovaries or testes from day 6 of the 21-day embryonic period. In ZZ males, Sertoli cells differentiate in the inner part of the gonad (medulla) and the outer cortex regresses. Conversely, the left female (ZW) gonad becomes an ovary with thickened cortex and vacuolated medulla, whereas the right gonad fails to elaborate a thickened cortex and regresses. Two hypotheses have been advanced for the mechanism of avian sex determination: Z dosage and dominant W. According to the former, sex is determined by the dosage of one or more Z-linked genes (higher in males), whereas the dominant W posits that the female sex chromosome carries a dominant-acting ovary/female determinant [4–6]. Although neither mechanism has so far been definitely proven, most evidence now favours the Z dosage hypothesis [7,8].

The traditional view of sexual development in birds and other vertebrates is that the gonads develop into either ovaries or testes during embryonic life and the gonads then release hormones to masculinize or feminize the brain and the rest of the body. However, this idea has been undermined by studies of gynandromorphic birds, which provide evidence that sex determination is cell autonomous in avians. Zhao *et al.* [9] examined three naturally occurring but rare gynandromorphic chickens, which are male on one side of the body and female on the other (Fig. 2). Remarkably, the male side had greater breast muscle, a wattle and spur on the leg, whereas the female side had smaller breast muscle, no wattle and no spur [9]. These birds were found to have mostly ZZ cells on the male side and ~ 50% ZW cells on the female side. The mechanism for this sexual mosaicism is thought to be due to failure of polar body exclusion during female meiosis, yielding a fertilized egg with both ZZ and ZW pronu-

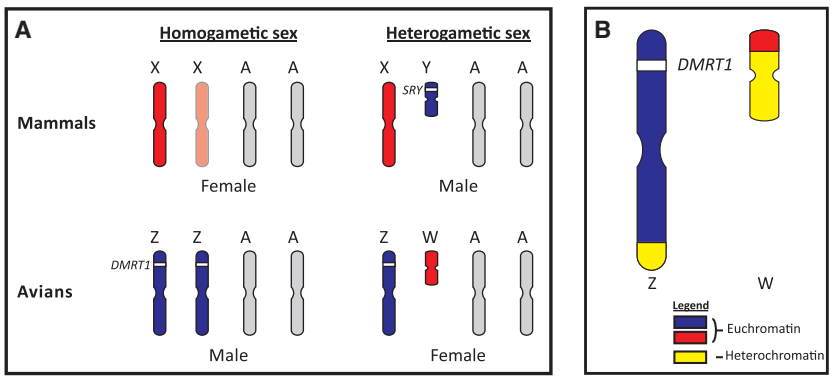

Fig. 1. Mammalian and avian sex chromosomes. (A) Schematic illustration of the sex chromosome systems used by mammalians and avians. Mammalian males are the heterogametic sex (XY) and females are the homogametic sex (XX). In avians, males are homogametic (ZZ) and females are heterogametic (ZW). The master testis-determining genes, *SRY* in mammals and *DMRT1* in avians, are represented by white bands on the Y and Z chromosomes, respectively. Dosage compensation only occurs in mammals where one X chromosome in females is randomly inactivated (represented by the faded chromosome). Autosomes are represented by grey chromosomes labelled 'A'. (B) Features of the Z and W chromosomes of modern avians. The euchromatic (blue in the Z, red in the W) and heterochromatic (yellow in both) regions are shown. Notably, the *DMRT1* gene is located exclusively on the Z chromosome in both ratites and nonratites.

Fig. 2. Gynandromorphic chicken. The left side of the chicken is female with brown colouration, small wattle and small leg spur. The right side of the chicken is male in colouration (predominantly white) and is characterized by the large wattle, large leg spur and greater breast musculature. Reprinted by permission from Macmillan Publishers Ltd. Zhao *et al.*, (2010), Nature, 464: 237–42. Copyright (2010).

clei and, hence a mosaic embryo with both male and female cells. The gynandromorphs cannot be explained by hormones, which would be expected to flow equally to both sides of the body. Zhao *et al.* [9] concluded that the sex differences from one side of the body to the other must have been determined directly by the sex chromosomes in each cell (cell autonomous). They provided further evidence that all cells 'know' their sex early in development by transplanting fluorescently labelled gonadal cells of one sex into the early gonad of the same or opposite sex (well prior to gonadal sex differentiation). When this was done for same-sex transplants, the donor and host cells integrated. However, when donor and host cells were of the opposite sex, no integration occurred [9]. Taken together, these data strongly suggest that sex in birds is determined by direct genetic factors operating in each cell throughout the body. This process appears to predate gonadal sex differentiation and the release of gonadal hormones. A possible alternative explanation for the tissue transplantation experiments could be that cells of one sex carry a cell-surface antigen that prevents integration with cells of the opposite sex, akin to the H-Y antigen of mammals [10]. Indeed, an H-W antigen occurs in female birds (ZW) [11]. However, this female-specific antigen appears to be induced by estrogen at the onset of gonadal sex differentiation (E6.5), and is not expressed at earlier stages [12–14]. Early expression of a similar antigen that prevents opposite sex tissue integration could be unrelated to later gonadal sex differentiation, or it could be part of the cell autonomous sex-determining process postulated by Zhao *et al.* [9].

Direct genetic effects of sex have also been inferred in a gynandromorphic zebra finch, which had male-type neural song circuitry on one side of the brain and female on the other [15]. The establishment of somatic sex independently of the gonads in birds is also supported by other studies, showing that sexually dimorphic gene expression occurs in embryos before the gonads have differentiated into testes or ovaries and hence, before sex-specific hormonal signalling [6,16]. In addition, transplantation studies in quail embryos have shown that male quails with female brains show female rather than male behaviour [17]. The authors of the gynandromorph studies do not preclude a supporting role for steroid hormones, such as testosterone and estrogen. Hormones must play some role in early sexual development, because genetically female chicken embryos will develop as male with testes if the estrogen-synthesizing enzyme, aromatase, is experimentally blocked very early in development (day 3–4) [18–20]. Nevertheless, these most recent findings show that 'sex determination' does not centre solely on the gonads in birds, but is perhaps best used to describe a genetic process that occurs throughout the whole embryo. Similar observations have been reported in mammals. For example, primary cell cultures of rat and mouse hypothalamus undergo sexual differentiation *in vitro* before the onset of sexually dimorphic gonadal secretions [21,22]. Furthermore, in marsupial mammals, the differentiation of a pouch in females or a scrotum in males predates gonad differentiation and is instead dependent on X chromosome dosage [23,24].

The authors of the gynandromorphic chicken study have suggested that the dosage of Z-linked genes might underlie sex determination throughout the avian body: males (ZZ) have two doses of all Z-linked genes, while females (ZW) have one (Fig. 1A). Hence, the 'Z transcriptome' could confer sexual identity to each cell in the body [9]. It is important to note in this context that there is no chromosome-wide dosage compensa-

tion mechanism in birds, as occurs for the X chromosome in mammals [25–29]. Hence, on average, male chickens have double the dosage of most Z-linked genes compared with females, and this might be the mechanism that determines whether a cell identifies as male or female. It is also worth noting that, in ratites (flightless birds), the Z and W sex chromosomes are near identical, carrying almost the same suite of genes, which would presumably undermine a sex-determining mechanism that relied upon overall Z chromosome transcript dosage. Hence, if sex is determined cell autonomously by the same mechanism in all birds, it may rely on one or a few Z-specific genes, or by a dominant W gene, present in both ratites and nonratites (flying birds). Regardless of whether sex is cell autonomous or hormonally influenced in birds, key genes must control development of the embryonic gonads into ovaries or testes as part of the sexual differentiation process. One such gene is *Double-sex and Mab-3 Related Transcription Factor #1* (*DMRT1*).

The *DMRT1* gene and testis differentiation

The chicken Z sex chromosome has over 680 known protein-coding genes, 49 novel genes and at least 45 noncoding RNA genes (http://www.ensembl.org/Gallus_gallus). Any of these genes could play a role in cell autonomous sex determination and/or downstream gonadal sex differentiation. The favoured candidate Z-linked gene controlling gonadal sex differentiation is *DMRT1*. This gene is conserved among vertebrate embryos and is more highly expressed in male gonads than female gonads, in the chicken embryo and in other groups, such as mammals, reptiles and fishes [30–33].

In avians, *DMRT1* is present on the Z and absent from the W sex chromosome of all birds, including, importantly, the flightless ratites. By contrast, most other Z-borne genes have orthologues on the W in ratites. *DMRT1* encodes a transcription factor with a zinc-finger like DNA-binding domain (the DM domain). It has been suggested that the higher dose and higher expression level of *DMRT1* in male (ZZ) chicken embryos triggers testicular development, wheresas a lower dose and lower expression are compatible with ovarian development [34]. Consistent with this idea, recent experimental knockdown of *DMRT1* in early chicken embryos resulted in the feminization of male gonads, affecting tissue organization, gene expression and germ cell distribution [7]. These findings support the Z dosage hypothesis for avian sex determination or at least, for gonadal sex development, because ZZ males would have a higher dosage of *DMRT1* which would initiate testicular differentiation and expression of the key conserved Sertoli cell differentiation factor, *SOX9* [35]. Further support for DM domain genes playing a decisive role in vertebrate gonadal development comes from the medaka fish (*Oryzias latipes*), in which *DMY/Dmrt1bY* operates as the master testis determinant [36–38]. Interestingly, a W-linked copy, *DM-W*, has been implicated in ovarian development in the African clawed toad (*Xenopus laevis*) and is thought to function by interfering with DMRT1 [39,40].

Is *DMRT1* the Z-linked factor responsible for sex determination in birds? This may be true for gonadal sex differentiation but not other tissues of the body (Fig. 3). In the embryonic gonads, a higher dose of *DMRT1* is required for proper testis formation and hence, the gene has a key role in the male gonad [7]. However, the cell transplantation experiments of Zhao

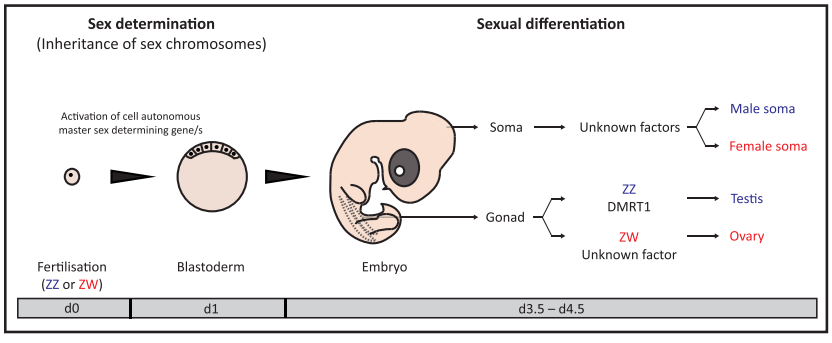

Fig. 3. Sex determination and sexual differentiation in the chicken model. Sex determination occurs in the early embryo (E0–E2) in a cell autonomous manner and is governed by the inheritance of sex chromosomes. Sexual differentiation of tissues such as the soma and gonads then occurs as development proceeds. Gonadal sex differentiation in ZZ males involves the *Dmrt1* gene while the master female sex differentiation gene remains unknown. Because sexual differentiation is at least partly cell autonomous, other currently unidentified sex-linked genes may control sex differentiation in the brain and other somatic tissues.

et al. [9] carried out at day 2 (stage 11/12) of development imply that the gonadal precursor cells have a sexual identity before the gonads form. *DMRT1* is more highly expressed in male urogenital tissues than female tissues as early as at least day 3.5 (stage 19), where it localizes to the coelomic epithelial cells overlying the mesonephric kidneys (CA Smith, pers obs). This is well prior to gonadal sex differentiation. If *DMRT1* is expressed even earlier, at day 2, it could be responsible for, or contribute to, gonadal sexual identity before morphological differentiation (day 6), in line with the findings of Zhao *et al*. [9]. Alternatively, another Z-linked gene, or a W-linked inhibiting factor, could lie upstream of *DMRT1* in the gonadal sex differentiation pathway [9]. The pathway conferring sexual identity outside the gonads, if cell autonomous, is unlikely to involve *DMRT1* which is not expressed outside the urogenital system. According to this second alternative, another (unknown) sex-linked gene is the sex-determining factor in birds and, in the gonads, this gene directly or indirectly influences sexually dimorphic DMRT1 expression. Therefore, if somatic sex is cell autonomous, another sex-linked gene is implicated. This could be any one (or more) of the 600 or so Z-linked genes mapped to the chicken Z sex chromosome, but, as mentioned above, the Z gene would presumably need to be absent from the ratite W. Most are not. Alternatively, sex could be controlled by a W-linked feminizing factor. Future studies should focus on expression profiling of non-gonadal tissues such as the brain to identify sex-linked regulatory mRNAs.

Conservation of the ovary-determining pathway in birds

According to the dominant W hypothesis, the W carries an ovary-determining gene that confers female development (analogous to the mammalian Y chromosome which carries the testis-determinant, *SRY*). However, no good candidate genes have been mapped to the W sex chromosome, which is heterochromatic and largely composed of repetitive DNA [27,41–43] (Fig. 1B). Some W-linked genes have been identified and some are expressed in the embryonic gonads, but there is no direct evidence of any role in ovary formation [44,45]. One well-characterized W-linked sequence is *HINTW* (also known as *WPKCI* and *ASW*) [44,46]. This gene encodes an aberrant histidine triad nucleotide-binding protein. It has been suggested that it might operate as a dominant negative, interfering with a Z-linked orthologue (called *HINTZ*) to direct female development [46,47]. However, misexpression of this gene in genetically male embryos (ZZ) does not perturb normal testis development [7]. Yamada *et al*. [48] carried out a detailed expression screen for novel W-linked genes that might be activated in early embryonic chicken gonads, but no new appealing candidate genes were identified.

FOXL2, *WNT4* and *RSPO1* have been proposed as candidate ovary-determining genes based on loss-of-function mutants in mammals. *FOXL2* mutations were identified as the underlying genetic cause of blepharophimosis/ptosis/epicanthus inversus syndrome (Online Mendelian Inheritance of Man: 605597; http://www.ncbi.nlm.nih.gov/omim), an autosomal disease that is associated with premature ovarian failure [49]. In further support of a role for *FOXL2* in ovary development, mutations affecting the levels of FOXL2 were shown to cause female-to-male sex reversal in goats with polled intersex syndrome [50]. Further analysis of *FOXL2* revealed that it encodes a forkhead/winged helix transcription factor that is only detectable in XX gonads and expressed during embryonic development, postnatally and in adulthood [51]. Subsequent studies of *Foxl2* mouse models have shown that knockout mice have phenotypes similar to blepharophimosis/ptosis/epicanthus inversus syndrome in humans and that ablation in adult ovaries leads to the transdifferentiation of granulosa cells to Sertoli-like cells [52,53]. Despite the somewhat different effects of *FOXL2* mutations observed in different mammals, it is clear that this gene has a role in the development and maintenance of the mammalian ovary. A conserved role for *FOXL2* is implied in the chicken embryo, where the gene is activated female-specifically just prior to gonadal sex differentiation (E5.5) [54,55]. The temporal and colocalization profiles suggest that FOXL2 activates aromatase during chicken ovarian development [55,56].

Another gene implicated in ovarian development is *WNT4*, which is upregulated in female gonads of chicken [57]. In mammals, *Wnt4* activates canonical β-catenin signalling, which is required for proper ovary formation [58,59]. Most recently, a novel activator of the Wnt/β-catenin signalling pathway known as *R-spondin 1* (*RSPO1*) was identified. In the chicken embryo, as in mammals, RSPO1 is female upregulated. *RSPO1* and *WNT4* are expressed in the cortex to outer medulla of the gonads, whereas FOXL2 and aromatase are present only in the medulla (at least at the time of sex differentiation) [57]. Thus, although there may be interaction between the two (for example, Wnt signalling influencing FOXL2 expression), the two pathways have distinct roles in ovarian development and it is hypothesized that experimental knockdown

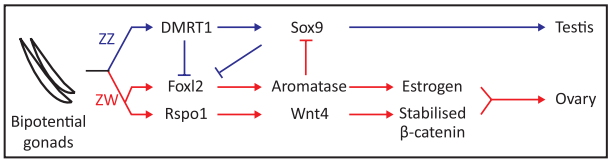

Fig. 4. Molecular factors hypothesized to be involved in avian sex determination and gonadal sex differentiation. Schematic model of the genetic cascades thought to control sex determination and differentiation of the embryonic gonads into testes (represented in blue) or ovaries (represented in red). Components and several aspects of this interplay are based on current knowledge in both avian and mammalian sex determination.

of both genetic cascades might induce testicular development in birds, as implied in masculinized *Foxl2*$^{-/-}$ *Wnt4*$^{-/-}$ double knockout mice [60].

Conclusion

The process of sex determination is essential to reproduction and is dependent on the proper development of the embryonic gonads. Significant progress in understanding the mechanism of avian sex determination has been made in recent years, leading to the identification of genes and regulatory networks that govern the fate of the gonads (Fig. 4). In the embryonic chicken gonad, Z-linked *DMRT1* is required for testis development and is likely to be the master regulator of testis differentiation. However, proof of this awaits overexpression studies in ZW embryos. We hypothesize that *DMRT1* activates *SOX9*, probably indirectly or with other factors, given that *DMRT1* is first expressed well before *SOX9* (day 3.5 versus day 6). Alternatively, high levels of *DMRT1* expression may act to inhibit the female pathway. In normal females (ZW), a lower level of *DMRT1* may be insufficient to repress ovarian development genes. If sexual fate of the embryonic chicken gonad depends on the dosage of Z-linked *DMRT1*, its regulation must be very important, because males have twice the dose of females, at least initially. Although DMRT1 is clearly important for testicular differentiation, cell transplantation studies suggest that other sex-linked genes may operate upstream of DMRT1 and cell autonomously to determine sex. DMRT1 is a likely target of these other undefined genes in the gonad, but the targets in other tissues of the avian body are completely unknown.

In females, the key ovary determinant is unclear, but it involves activation of both *FOXL2* and *RSPO1*. In this sense, avian gonadal (ovary) development is conserved with mammals. Several outstanding questions remain. What is the nature of the sex determinant that operates cell autonomously throughout the body in early embryos? What activates *DMRT1* and what are its target genes? These will be fruitful areas of research in the coming years.

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