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Sex determination and gonadal sex differentiation in the chicken model

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ABSTRACT Our understanding of avian sex determination and gonadal development is derived primarily from the studies in the chicken. Analysis of gynandromorphic chickens and experimental chimeras indicate that sexual phenotype is at least partly cell autonomous in the chicken, with sexually dimorphic gene expression occurring in different tissue and different stages. Gonadal sex differentiation is just one of the many manifestations of sexual phenotype. As in other birds, the chicken has a ZZ male: ZW female sex chromosome system, in which the male is the homogametic sex. Most evidence favours a Z chromosome dosage mechanism underling chicken sex determination, with little evidence of a role for the W chromosome. Indeed, the W appears to harbour a small number of genes that are un-related to sexual development, but have been retained because they are dosage sensitive factors. As global Z dosage compensation is absent in birds, Z-linked genes may direct sexual development in different tissues (males having on average 1.5 to 2 times the expression level of females). In the embryonic gonads, the Z-linked *DMRT1* gene plays a key role in testis development. Beyond the gonads, other combinations of Z-linked genes may govern sexual development, together with a role for sex steroid hormones. Gonadal DMRT1 is thought to activate other players in testis development, namely *SOX9* and *AMH,* and the recently identified *HEMGN* gene. DMRT1 also represses ovarian pathway genes, such as *FOXL2* and *CYP19A1*. A lower level of DMRT1 expression in the female gonads is compatible with activation of the ovarian pathway. Some outstanding questions include how the key testis and ovary genes, *DMRT1* and *FOXL2,* are regulated. In addition, confirmation of the central role of these genes awaits genome editing approaches.

KEY WORDS: *chicken, gonad, sex determination, sexual differentiation, embryonic*

Introduction

The chicken (*Gallus gallus domesticus*) is a scientifically and commercially important species. It has been used as an accessible model organism for researchers for over 100 years and it is a major food source for the human population (Doran *et al.*, 2016). The global poultry industry currently seeks methods of modulating sexual development in chickens. In the egg industry, in particular, only female birds are required and males are usually culled, a significant animal welfare and economic issue (Doran *et al.*, 2017). The ability to generate monosex lines of birds (e.g., all female) would be of significant value to the poultry industry. Efforts to modulate sex in chickens depends upon a sound knowledge of normal sex determination and sexual development. Recent years have seen some major advances in our understanding of chicken sex determination (Schmid *et al.*, 2015). This review will describe the current knowledge around sex determination in the chicken, with particular emphasis of sexual differentiation of the gonads, and the role played by key transcription factors, signalling molecules and hormones. Three major advances have been made in the past 10 years; (1) the finding that sexual phenotype is at least partly cell autonomous in chicken, in the gonads and throughout the embryo (Clinton *et al.*, 2012, Zhao *et al.*, 2010) (2) definition of the gene content of the curious W sex chromosome and (3) the discovery of the likely master genetic switch for testis development, *DMRT1* (doublesex and mab-3-related transcription factor 1) (Lambeth *et al.*, 2014, Major and Smith,

Abbreviations used in this paper: DMRT1, doublesex and mab-3-related transcription factor 1; HH, Hamburger and Hamilton stage; PGC, primordial germ cell.

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2016, Raymond *et al.*, 1999, Shan *et al.*, 2000, Smith *et al.*, 1999a, Smith *et al.*, 2009a).

Sex determination and sexual development

Fundamentally, sex is determined at fertilization by the differential inheritance of the sex chromosomes. In chicken and other birds, male is ZZ and female is ZW. Avian sex chromosomes are not homologous to those of mammals, having evolved from a different pair of autosomes in reptilian ancestors (Graves, 2016, Warren *et al.*, 2017). The traditional model of chicken sex determination follows that espoused for other vertebrates (Jost *et al.*, 1973); genes on one or both of the sex chromosomes direct differentiation of the embryonic gonads into either ovary (ZW) or testis (ZZ), and steroid hormones secreted from the gonads then feminise or masculinise other parts of the body. However, this "gonad-centric" view of sex looks increasingly antiquated, as several lines of evidence indicate that direct genetic effects can play a major cell autonomous role in sexual development, both in birds and other organisms (Agate *et al.*, 2003, Arnold, 1996, Arnold, 2012, Arnold *et al.*, 2013). It is now no longer appropriate to refer to gonadal sex differentiation in birds or mammals as "sex determination." Gonadogenesis is properly viewed as one of many manifestations of "sexual differentiation".

Sexual differentiation involves both direct cell autonomous and indirect hormonal mechanisms, and aspects of sexual differentiation in the embryo can precede those in the gonads. In birds, several lines of evidence support the notion of at least partial cell autonomous sexual development. Zhao and colleagues reported three rare gynandromorphic chickens that are bilateral sex chimeras – male on one side of the body and female on the other (Fig. 1) (Zhao *et al.*, 2010). Such unusual birds are unlikely to derive from a hormonal mechanism, as hormones would flow to both sides of the body. They are also unlikely to derive from a mutation in a ZW or ZZ embryo at the two-cell stage of development as the birds had both ZZ and ZW cells (Fig. 1B). The "male" side was predominantly ZZ, while the "female" side had at least 50% ZW cells (Fig. 1B and C). The authors termed this phenomenon CASI (Cell Autonomous Sex Identity) and have noted that such a phenomenon has been also reported in other organisms (Clinton *et al.*, 2012). These gynandromorphic birds support the notion that cells in the body of the chicken have an innate sexual phenotype, involving direct genetic effects. Zhao *et al.,* supported this idea by producing cross-sex embryonic chimeras. This was achieved by transplanting female pre-gonadal mesodermal tissue into male hosts and vice versa, prior to the time of gonadal sex differentiation. Based on the expression of markers, the transplanted tissue differentiated into the sex of the donor, not the host (Zhao *et al.*, 2010). This again supported direct cell autonomous sexual development.

These findings are consistent with the finding that sexually dimorphic gene expression in chicken embryos can occur prior to the time of gonadal sex differentiation and hence predate any sexually dimorphic hormonal output (Ayers *et al.*, 2013a, Lin *et al.*, 2010, Major and Smith, 2016, O'Neill *et al.*, 2000, Scholz *et al.*, 2006, Zhang *et al.*, 2010, Zhao *et al.*, 2010). In a comparable experiment to that of Zhao *et al.*, Maekawa *et al.,* (2013) switched primordial brains between ZZ and ZW chicken embryos before gonadal sex differentiation (and vice versa) and raised birds to sexual maturity. The transplanted donor brains retain the neuroendocrine features of the donor (Maekawa *et al.*, 2013). This is in agreement with previous data pointing to an intrinsic sexual identity of brain tissues, at least partly independent of gonadal sex hormones (Arnold, 1996, Wade and Arnold, 1996, Wade *et al.*, 1997, Wade *et al.*, 1996). Most recently, our own laboratory produced transgenic chicken constitutively expressing the *CYP19A1* gene, which synthesises oestrogen and can feminise male gonads, yet hatched birds maintained a predominantly male phenotype (Lambeth *et al.*, 2016b). Taken together, these data indicate that direct genetic factors have a major role in regulating sexual differentiation in the chicken. There is nevertheless a role for gonad derived sex steroid hormones in avian sexual differentiation, both in the gonads and in extragonadal tissues. Oestrogen is essential for chicken ovarian development (see below for more details). Blocking oestrogen synthesis can have a potent masculinising effect upon genetically female birds (Elbrecht and Smith, 1992a, Lambeth *et al.*, 2013, Vaillant *et al.*, 2001), while over-expression of *CYP19A1* or the administration of oestrogen can feminise males, albeit transiently (Lambeth *et al.*, 2013, Scheib, 1983). Meanwhile gonadectomy can also alter sexual features such as plumage and behaviour (Lambeth and Smith, 2012, Owens and Short, 1995). Clearly, sexual differentiation in the chicken must have both direct cell autonomous and hormonal input.

In the case of direct cell autonomous factors regulating sexual differentiation, different tissues may respond to different combinations of sex-linked factors, that could be either Z- or W-linked. The large chicken Z chromosome

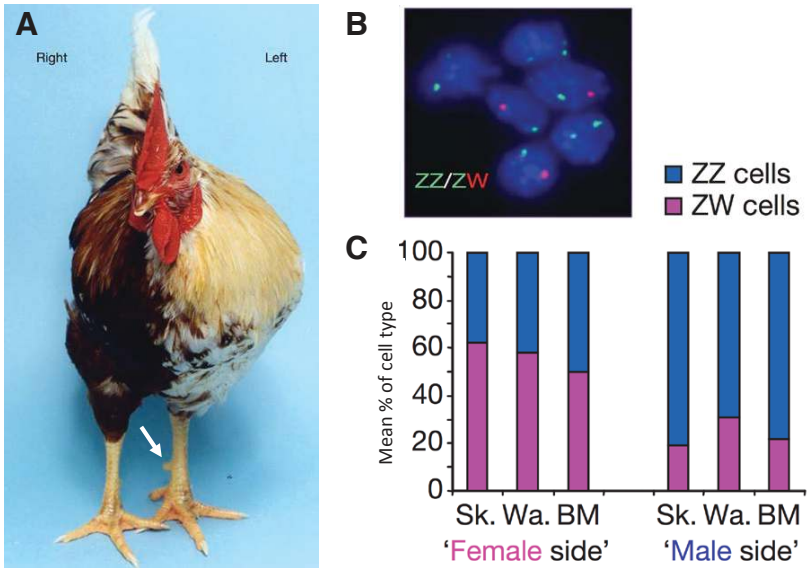

Fig. 1. Distribution of male and female cells in a gynandromorphic chicken. (A) *A naturally occurring gynandromorphic chicken, with sex-linked feather colouring. The right side is female, with brown feathers, smaller leg, wattle and breast muscle. The left side is male, with pale feathering and larger wattle, breast muscle and a spur in a larger leg (white arrow).* **(B)** *FISH analysis of sex chromosomes in gynandromorph blood cells. Interphase nuclei, showing a mix of both ZZ and ZW cells.* **(C)** *Mean relative proportions of ZZ and ZW cells in tissues from "male" and "female" sides of gynandromorph birds. Sk, skin; Wa, wattle; BM, breast muscle. Reproduced from Zhao* et al., *(2010), with permissions from Nature Publishing Group.*

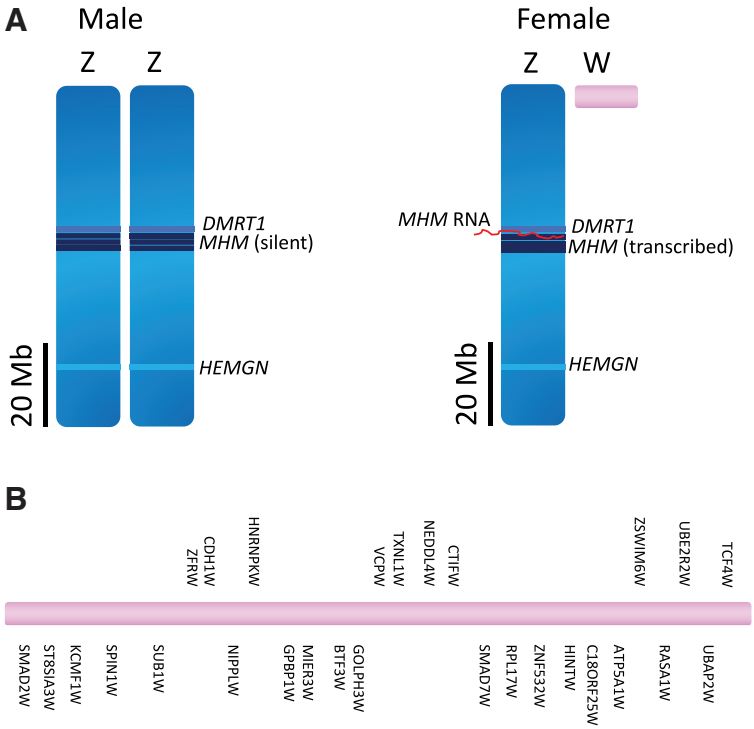

Fig. 2. Schematic view of chicken sex chromosomes and sex determining genes. (A) *The large Z chromosome (82.3 Mb) is drawn to scale next to the degenerate W chromosome (7 Mb). Male (ZZ) chickens have two copies of* DMRT1 *and* HEMGN*, while the female (ZW) only has one. The* MHM *locus is transcribed from the single Z in the female and may play a role in local dosage, and epigenetic regulation of* DMRT1 *in the female.* **(B)** *Location and orientation of the 28 protein coding genes that are located on the W chromosome. Modified from Bellott* et al.*, (2017), with permissions from Nature Publishing Group.*

harbours over 1000 genes, most of which are "house-keeping" genes unrelated to sex (Schmid *et al.*, 2015). The chicken W sex chromosome is a severely degraded copy of the Z chromosome, with perhaps as few as 28 genes having remained over evolution (Fig. 2B) (Ayers *et al.*, 2013a, Bellott *et al.*, 2017, Mank and Ellegren, 2007, Moghadam *et al.*, 2012). This sets up a dosage inequality of most Z-linked genes between the sexes. In the context of cell autonomous sexual development, it is particularly noteworthy that birds lack a system of global Z chromosome dosage compensation akin to mammalian X inactivation (Arnold *et al.*, 2008, Ellegren, 2011, Ellegren *et al.*, 2007, Itoh *et al.*, 2007, Kuroda *et al.*, 2001, Melamed and Arnold, 2007, Naurin *et al.*, 2012, Wang *et al.*, 2017). In the chicken, Z-linked genes are expressed on average at 1.4 -1.8 times more highly in males (ZZ) compared to females (ZW), across various tissues (Arnold and Itoh, 2011, McQueen and Clinton, 2009, Wright *et al.*, 2012). There is some local equalisation of gene dosage, but this is gene-specific, and involves Z-linked genes that are dosage sensitive and for which a certain dose of expression is critical (Mank and Ellegren, 2009, McQueen and Clinton, 2009, Zimmer *et al.*, 2016). The overall Z gene dosage inequality between the sexes exists in gonadal and non-gonadal cells prior to gonadal sex differentiation into ovaries or testes (Ellegren *et al.*, 2007) and so could provide a mechanism for cell autonomous sexual development. Alternatively, W-linked genes could be a source of sexual dimorphic gene expression, but recent studies show that the few genes present on the chicken W chromosome are very highly homologous to partners on the Z (so-called "gametologues") and are expressed at similar levels (Ayers *et al.*, 2013a). These W genes are likely to be dosage sensitive factors that have been retained on the otherwise degenerate chicken W to match expression between females and males (Bellott *et al.*, 2017).

One region of the chicken Z sex chromosome has a concentration of genes that do show dosage compensation, and these genes lie adjacent to a Z-linked locus called *MHM* (Male HyperMethylated). This 2.2kb repeat sequence is hypermethylated and silent in male cells (ZZ) but is hypomethylated on open chromatin and transcribed into a long non-coding RNA in female cells (ZW) (Fig. 2A) (Teranishi *et al.*, 2001). Intriguingly, *MHM* lncRNA coats the female Z chromosome in close proximity to a subset of genes that are compensated (up-regulated in females). *MHM* is ostensibly similar to *XIST* in mammals, which is a non-coding RNA that mediates X inactivation (Briggs and Reijo Pera, 2014). However, in the case of *MHM*, the neighbouring female genes are up-regulated, and the associated histone H4K16 is hyperacetylated, an epigenetic mark of active gene up-regulation (Bisoni *et al.*, 2005). *MHM* may thus play a role in local dosage compensation, elevating Z-linked gene expression in females to levels comparable to that of males. However, the sequence although it appears to be limited to Galliform birds (such as chickens and turkey), and not all avians (Itoh *et al.*, 2010, Wright *et al.*, 2015). The likely avian testis-determinant *DMRT1* (see below), is located very close to the site of *MHM* binding, and indeed both are located on the same loop in lampbrush preparations of the Z chromosome (Teranishi *et al.*, 2001). This had led to the suggestion that *MHM* could play a role in gonadal sex differentiation by contributing to epigenetic repression of *DMRT1* expression female gonadal cells (Caetano *et al.*, 2014, Roeszler *et al.*, 2012, Teranishi *et al.*, 2001, Yang *et al.*, 2016, Yang *et al.*, 2011, Yang *et al.*, 2010). Interestingly, Itoh and colleagues reported an asymmetric effect of the demethylating agent 5-aza-cytidine on *MHM* expression from the two Z chromosomes of male cells, with one expressing more than the other (Itoh *et al.*, 2011). This suggests an inequality of the *MHM* methylation status between the two Z sex chromosomes, but the functional significance of this observation is unclear. In the chicken, *MHM* expression begins at the time of fertilization in ZW zygote. The role of *MHM* may be linked to either local dosage compensation or sex determination, or both. Global over-expression of *MHM* causes developmental abnormalities in chicken embryos that are not sex-specific, and an apparent reduction in *DMRT1* gonadal expression (Roeszler *et al.*, 2012). The exact function of this sequence could be further clarified by knockdown or knockout (CRISPR/Cas9) approaches (Woodcock *et al.*, 2017). If it indeed plays an essential role in local dosage compensation, one might predict that loss of *MHM* expression would cause lethality of female embryos. If it has a role in repressing *DMRT1* expression in ZW cells, loss of *MHM* could lead to elevated *DMRT1* and testicular development.

Chicken sex chromosomes

The Z and/or W sex chromosomes of the chicken must harbor one or more sex-determining genes. The 82.3 Mb chicken Z chromosome is large and contains over 1000 genes, many with

"house-keeping" functions (Handley *et al.*, 2004). There are 884 protein-coding sequences and some 348 non-coding genes (microRNAs and long non-coding sequences) (Bellott *et al.*, 2010, Ellegren, 2011). Due to the absence of global dosage compensation and hence potential expression inequality between ZZ and ZW cells, any of these genes could serve a sex-determining role in a particular tissue (Ayers *et al.*, 2013b). The Z sex chromosome has been strongly "masculinised" over evolution (enriched for male-biased gene expression) (Kaiser and Ellegren, 2006, Mank and Ellegren, 2009, Storchova and Divina, 2006, Wright *et al.*, 2012) and there has also been an accumulation of genes related to sex and reproduction on the Z (Ellegren, 2011, Mank *et al.*, 2007, Morkovsky *et al.*, 2010, Naurin *et al.*, 2012). This includes two important genes expressed in the developing testis, *DMRT1* and *HEMGN* (discussed below). These observations suggest that it is the Z, rather than the W, that plays a central role in chicken sex determination. Indeed, the W chromosome lacks an obvious candidate gene that could be sex-determining. As noted above, the chicken W sex chromosome is a smaller degraded version of the Z, with perhaps as few as 28 bona fide genes. (Fig. 2B). At 7 Mb, the chicken W chromosome is 6% of the size of the Z chromosome. Most of the W chromosome is heterochromatic, with a smaller euchromatic region harbouring protein-coding genes. The most recent build of the chicken W chromosome (galGal5) annotates 25 protein-coding genes and around 116 non-coding RNAs (Bellott *et al.*, 2017, Warren *et al.*, 2017). In theory, any of these could play a sex-determining role, as for any non-dosage compensated Z-linked genes described above. However, we have previously found that the 27 W-linked genes have partners on the Z sex chromosome to which they are they are highly homologous (over 90% in most cases) (Ayers *et al.*, 2013a, Warren *et al.*, 2017). In the chicken (and in the collared flycatcher), the combined expression levels of the Z and W homologues in females are comparable to the expression levels from the two Z chromosomes in males, and expression occurs broadly across embryonic and/or adult tissues (Ayers *et al.*, 2013a, Smeds *et al.*, 2015) (Fig. 3). These features make these genes unlikely sex determinants (Bellott *et al.*, 2017).

It has been proposed that the small number of single copy dosage sensitive genes retained on the chicken W have important roles in development. Indeed, these genes have predicted UniProt annotations associated with fundamental cellular process, such as transcription, translation, protein degradation, chromatin modification and signal transduction (Bellott *et al.*, 2017). Only one W-linked gene is significantly divergent in sequence from its Z homologue, *HINTW* (Hori *et al.*, 2000), and this gene has been amplified into a multicopy family containing approximately 40 copies on chicken W chromosome (Bellott *et al.*, 2017). *HINTW* is conserved among volant (flying) birds and is expressed widely in chicken embryos (Hori *et al.*, 2000, O'Neill *et al.*, 2000). These features have made *HINTW* a candidate female or ovary determinant. The Z homologue encodes a histidine triad nucleotide binding protein of the HIT family, however, HINTW is aberrant in that it specifically lacks the key catalytic domain required for the protein to function (Brenner, 2002, Moriyama *et al.*, 2006, Parks *et al.*, 2004). As such it has been suggested that HINTW could act as a dominant negative in avian sex determination, blocking the male-promoting function of HINTZ (Ayers *et al.*, 2013a, Bellott *et al.*, 2017, Brenner *et al.*, 1999, Moghadam *et al.*, 2012, Pace and Brenner, 2003). However, despite these findings, over-expression of HINTW does not induce feminisation of ZZ chicken embryos (Smith *et al.*, 2009b). However, recent evidence has shown that experimental female to male sex reversal using the aromatase inhibitor fadrozole, does not affect the high level of *HINTW* RNA in chicken gonads. Furthermore, antibodies generated and validated against chicken HINTW fail to detect endogenous HINTW protein in chicken gonads. Additionally, while *HINTW* RNA can be detected in chicken and zebra finch gonads, no homologue appears to be present in the emu (Hirst *et al.*, 2017b). Taken together, this data undermines the role for *HINTW* as the W-linked female sex determining gene, and reinforces a role for gene dosage of Z-linked *DMRT1* as the mechanism for sex-determination in birds (discussed below).

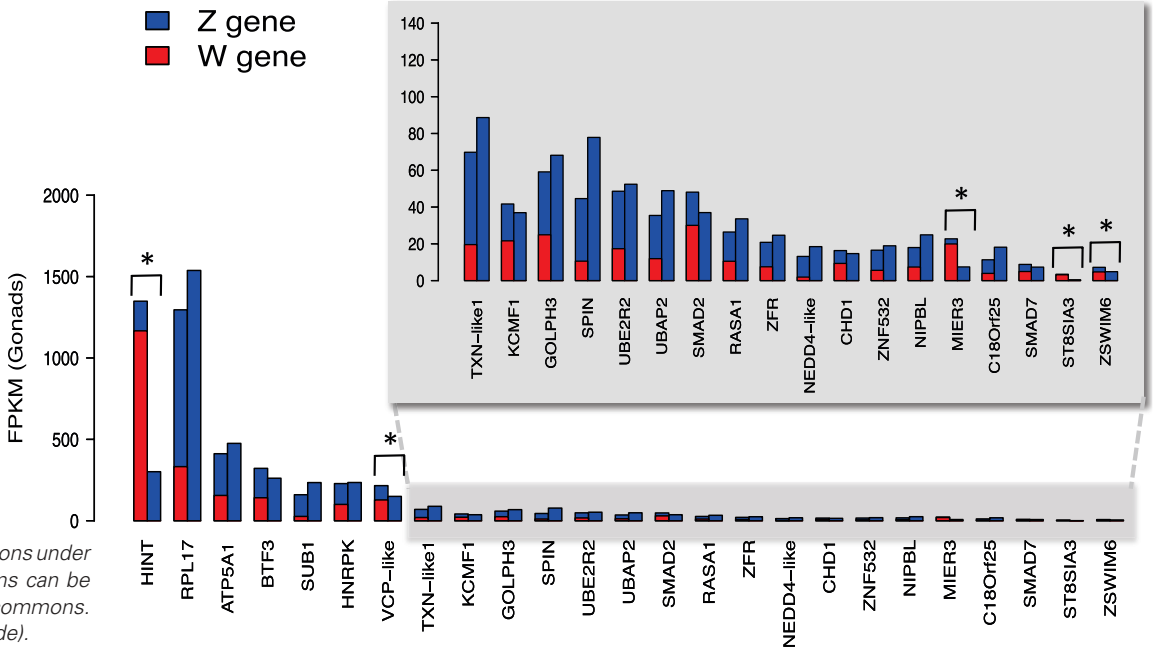

Fig. 3. Expression levels of W/Z gametologue pairs in the chicken gonad. *Expression of W-linked genes (red) compared to their Z-linked gametologues (blue) in chicken gonads. The total combined expression of gametologue pairs is shown for females (left bar of pair) and males (right bar of pair). The shaded data (inset) are shown on an adjusted FPKM scale. Genes with significantly different expression between the sexes are identified (* P <0.01). Reproduced from Ayers* et al., *(2013a), with permissions under CC-BY-4.0; the license terms can be found at: (https://creativecommons.org/licenses/by/4.0/legalcode).*

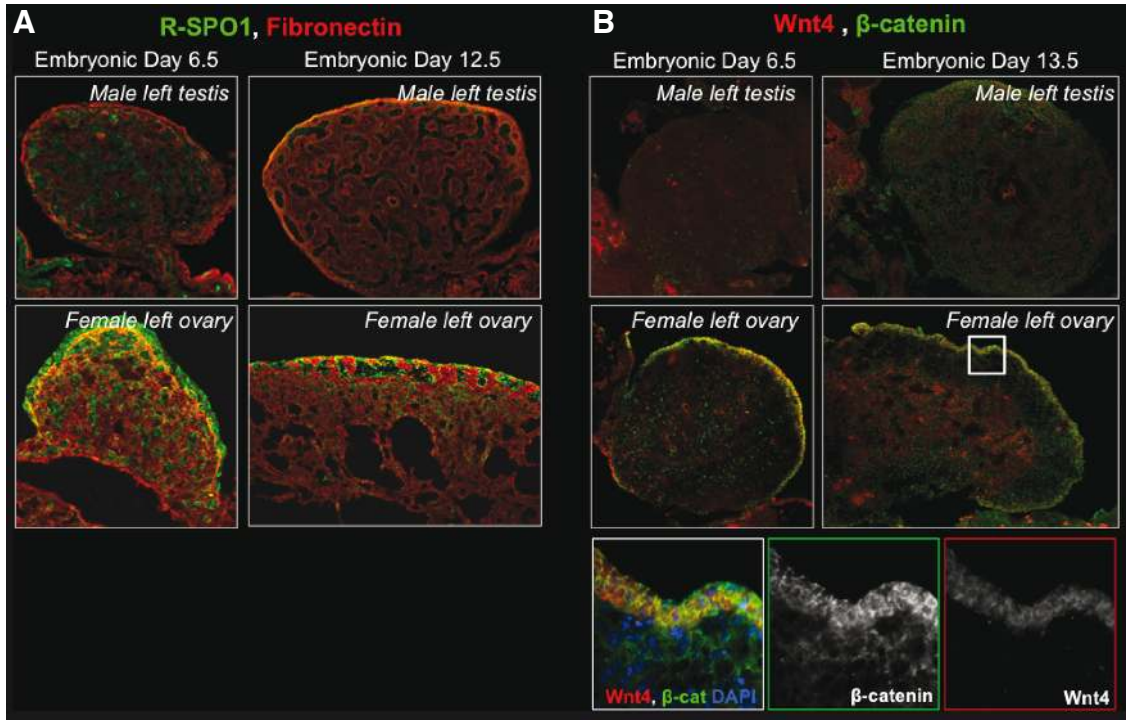

Fig. 4. Expression of R-spondin 1, WNT4 and β-catenin in the chicken embryonic ovary. (A) *Detection of RSPO1 protein in embryonic chicken gonads, using a chicken RSPO1-specific antibody raised in rabbits (green). RSPO1 is expressed in the developing ovarian cortex at E6.5 and E12.5, but is lowly expressed in the testis. Fibronectin (red) delimits the medulla from the cortex.* **(B)** *β-catenin (green) and WNT4 (red) expression in developing chicken gonads. Both proteins are highly expressed in the ovarian cortex from early gonadal stages, but not expressed in the testis. WNT4 is also upregulated in the inner medulla at later stages. Reproduced from Ayers* et al., *(2013b), with permissions from S. Karger AG, Basel.*

Embryonic development of the gonads

The embryonic urogenital system arises from the intermediate mesoderm at approximately day 3 (HH18) of embryonic development, and is marked by the thickening of the coelomic epithelium ventral to the mesonephros. At the same time, primordial germ cells (PGCs) migrate from the germinal crescent into the gonads via the blood stream (Ginsburg and Eyal-Giladi, 1987). Prior to differentiation at E4.5 (HH26), the undifferentiated gonad consists of a thin outer cortical layer overlying an inner medulla.

Several genes have been shown to be important for these initial stages of gonad differentiation. Two of the earliest genes expressed are *Steroidogenic Factor-1* (*SF1*) and *GATA4*, reviewed in Piprek *et al.,* (2016). *GATA4* is expressed in the bipotential gonad in chicken in both sexes at E4 (HH24) (Oreal *et al*., 2002), and it is also expressed in the earliest stages of gonad differentiation in mice (Viger *et al*., 1998). Conditional mutant mice lacking *Gata4* fail to form gonads, as there is no thickening of the coelomic epithelium to form the genital ridges (Hu *et al*., 2013). SF1 is not expressed in the coelomic epithelium of these mice suggesting that GATA4 is upstream of SF1 in initiating the formation of the gonadal primordium (Hu *et al*., 2013).

Sf1 is initially expressed in the undifferentiated gonads of both male and female mice, and its expression is maintained during testis differentiation but is down-regulated during ovarian differentiation (Ikeda *et al*., 1994). Consistent with this early expression, null mice lacking *Sf1* fail to develop gonads indicating that *Sf1* is essential for the formation of the gonadal primordium in both sexes (Luo *et al*., 1994). Chicken *SF1* is also observed in both sexes prior to gonadal differentiation at E5.5 (HH28), but dissimilar to that seen in the mouse, *SF1* expression is then up-regulated in developing ovaries after the onset of differentiation (E7.5 HH32) (Smith *et al*., 1999b, Smith *et al*., 1999c). This suggests a conserved role for SF1 in early gonad development across vertebrates, but suggests that later in development the role of SF1 in birds and mammals varies.

The differentiation of the bipotential gonad into either a testis or ovary occurs at approximately E6-6.5 (HH29-HH30) of chicken development. In males (ZZ), the cortex of the developing testis remains as a thin epithelial layer, while the gonadal medulla differentiates in seminiferous cords, which enclose the pre-Sertoli cells and PGCs. The pre-Sertoli cells produce anti-Müllerian hormone (AMH), which contributes to the regression of Müllerian ducts (which are the embryonic oviducts), and subsequently the testosterone-producing Leydig cells differentiate and reside in the mesenchyme outside of the seminiferous cords. In the male, PGCs become enclosed within the developing seminiferous cords and undergo mitotic arrest, entering meiosis only after hatching.

The differentiation of the ovary is characterised by the thickening of the outer cortex and the accumulation of PGCs within it (Carlon and Stahl, 1985). However, this process is asymmetrical as only the cortex of the left gonad proliferates while the right gonad regresses. This asymmetry is mediated by expression of the transcription factor *PITX2* in the left gonad specifically, which regulates expression of the oestrogen receptor-α and directs cell proliferation and differentiation of both somatic cells and PGCs within the left gonad (Guioli and Lovell-Badge, 2007, Ishimaru *et al*., 2008, Rodriguez-Leon *et al*., 2008). PGCs in the left medulla and right gonad, however, do not undergo meiosis due to the lack of retinoic acid (Smith *et al*., 2008a), These cells were originally thought to undergo apoptosis (Ukeshima, 1996), however, more recent analysis of PGCs in the right gonad and left medullar indicate that they arrest in meiosis and do not undergo apoptosis until after hatching (de Melo Bernardo *et al*., 2015).

The thickened cortex of the left gonad contains both somatic cells as well as proliferating PGCs. From E9 (HH35) these PGCs begin to undergo folliculogenesis via synchronous rounds of mitosis and

meiosis to form germ cell nests. The granulosa and thecal cells that enclose the germ cells are derived from somatic cells from either the cortex or from medullary cells that migrate from just beneath the cortex. Development of the functional left ovary is completed after hatching with the formation of primordial follicles that are arrested in the diplotene phase of prophase I. In the underlying the cortex of both the left and right gonads, the medullary cords become vacuolated and form structures referred to as lacunae.

Molecular mechanisms underlying female gonad differentiation

Two developmental pathways are thought to act in parallel to determine ovarian development; the first is the FOXL2/Aromatase/Oestrogen pathway. Oestrogen is an absolute requirement for ovarian development as it essential for the development of the ovaries (Elbrecht and Smith, 1992b, Vaillant *et al.*, 2001), and for the acquisition of secondary sexual characteristics in the adult female bird. Early studies describing the effect of exogenously applied hormones found that treatment of male (ZZ) birds with oestrogen, prior to gonadal differentiation, results in the transient feminization of the male left gonad and the formation an ovary or ovo-testis, while treatment of female (ZW) birds with anti-oestrogens perturbed the development of ovarian structures (Scheib, 1983).

The productions of oestrogens in the developing female (ZW) gonad is determined by the temporal and sex-specific expression of the enzymes involved in steroidogenesis (Bruggeman *et al.*, 2002). The enzymes involved in the upstream steps of the steroidogenesis pathway are expressed in the gonadal medulla of both sexes (Nakabayashi *et al.*, 1998), However, aromatase (CYP19A1) and 17β-hydroxysteroid dehydrogenase (17β-HSD), which are involved in the final steps of the conversion of androgen substrates into the active oestrogens, oestrone and 17β-oestradiol, are only expressed in the medulla of female (ZW) gonads (Nakabayashi *et al.*, 1998, Nishikimi *et al.*, 2000, Smith *et al.*, 1997, Smith *et al.*, 2005).

The aromatase protein is detected only in the female gonads from E6 (HH29) onwards and its expression increases during gonad differentiation (Smith *et al.*, 1997, Smith *et al.*, 2005). Likewise, 17β-HSD is expressed in the developing gonads of females, but not males (Nakabayashi *et al.*, 1998, Wajima *et al.*, 1999). As such, oestradiol is never detectable in male gonads and is detected in female gonads from E9 onwards (Imataka *et al.*, 1989). Underscoring the importance of aromatase in the synthesis of oestrogens, aromatase inhibitors, such as fadrozole, induce female-to-male sex reversal in (ZW) females when applied prior to, or during sexual differentiation (Abinawanto *et al.*, 1996, Burke and Henry, 1999, Elbrecht and Smith, 1992b, Hudson *et al.*, 2005, Smith *et al.*, 2003, Vaillant *et al.*, 2001, Wartenberg *et al.*, 1992). Furthermore, the addition of oestrogen rescues fadrozole induced- sex reversal (Elbrecht and Smith, 1992), demonstrating the absolute requirement for oestrogen in the development of the ovary.

In addition to enzymes responsible for oestrogen synthesis, several other genes have been implicated in sex determination in female birds. Numerous studies have indicated that the forkhead transcription factor FOXL2 is an essential player in ovarian development and maintenance in many species including fish, birds and mammals (Loffler *et al.*, 2003, Pisarska *et al.*, 2011, Wang *et al.*, 2004). FOXL2 is expressed in an ovary-specific manner at the time of gonadal sex differentiation in all vertebrates that have been examined. In the chicken, the onset of *FOXL2* expression is around E5.7 (HH28), just prior to aromatase and the first signs of ovarian differentiation at E6 (HH29), furthermore FOXL2 and aromatase are co-expressed within cells in the medulla of female gonads (Govoroun *et al.*, 2004). It is therefore thought that FOXL2 controls aromatase

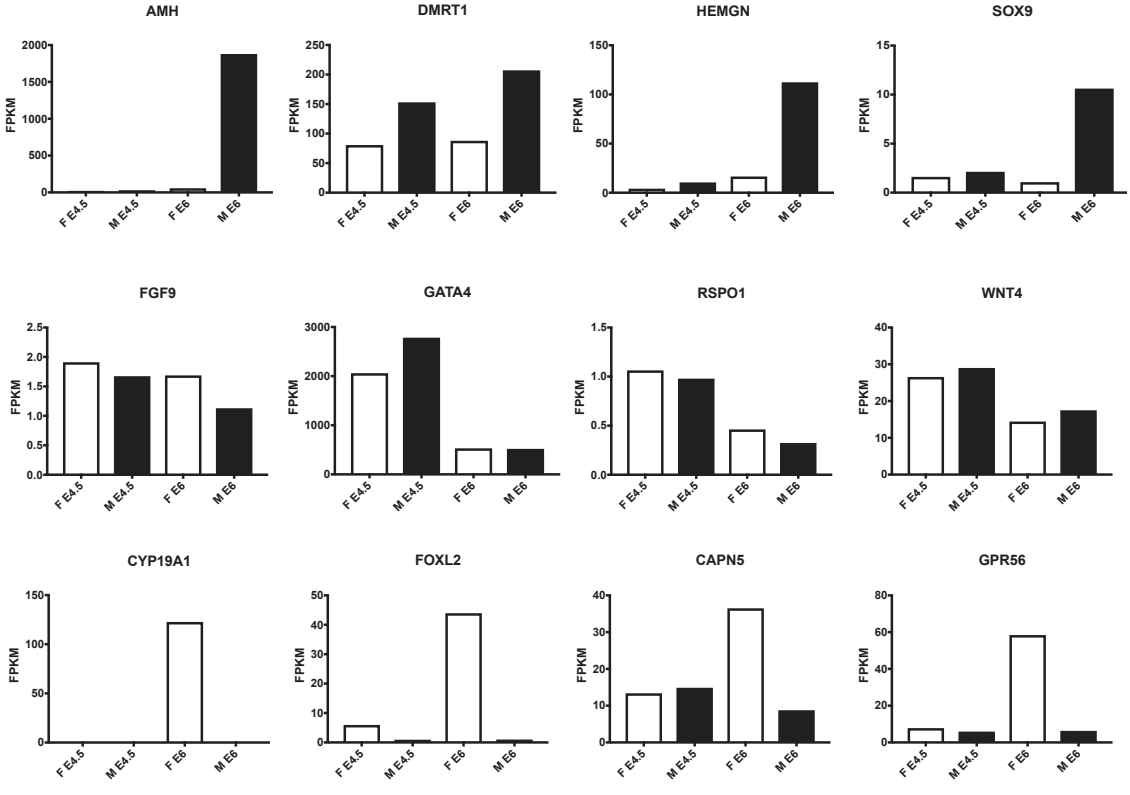

Fig. 5. The expression of known or putative sex-determining genes in the female and male gonads at E4.5 and E6. *RNA-sequencing data demonstrating the male or female biased expression of known and putative sex-determining genes. Expression is measured in FPKM (Fragments Per Kilobase of exon per Million fragments mapped); data obtained from Ayers* et al., *(2015b).*

transcription during differentiation of female gonads in the chicken, as has been previously shown for mammals (Bentsi-Barnes *et al.*, 2010, Fleming *et al.*, 2010, Pannetier *et al.*, 2006).

Consistent with this, *in vitro* analysis has demonstrated that FOXL2 can bind to a highly conserved putative forkhead element in the aromatase promoter to activate transcription (Fleming *et al.*, 2010). While it is not yet known whether FOXL2 can directly control aromatase expression (and therefore oestrogen levels) *in vivo*, aromatase inhibitors cause a reduction in *FOXL2* levels in the female gonads, suggesting a feedback regulatory loop exists between these two genes (Hudson *et al.*, 2005). Whether this is due to the loss of oestrogen synthesis causing a reduction in the expression of *FOXL2*. Or whether activation of the male sex determining pathway, and expression of male-specific genes such as *SOX9*, may result if the downregulation of *FOXL2*. As such the exact mechanism which underlies the interplay between these genes remains to be elucidated.

In mammals, the canonical WNT4/β-catenin signalling pathway has been shown to be a key regulator of ovarian development (Biason-Lauber and Konrad, 2008, Liu *et al.*, 2010). In mice, *Wnt4* is initially expressed in the gonad in both sexes but becomes restricted to the female (XX) gonads later in development (Vainio *et al.*, 1999). Loss of *Wnt4* in mice causes partial masculinization of the female (XX) gonads, with the loss of the Müllerian duct and concomitant retention of the Wolffian duct, and the ectopic activation of the testosterone synthesis pathway (Vainio *et al.*, 1999). Similarly, loss of β-catenin in female (XX) gonads also results in masculinization of the ovary with the formation of the testis specific coelomic vessel and androgen expressing cells, and the loss of female germ cells (Liu *et al.*, 2009). Mutations in *WNT4* in humans also cause various degrees of female to male sex reversal (Biason-Lauber *et al.*, 2007, Biason-Lauber *et al.*, 2004, Mandel *et al.*, 2008) indicating the importance of this pathway in the development of the ovary. In the chicken, *WNT4* is expressed in the bi-potential gonads of both sexes at E4.5 (HH26), during sexual differentiation from E6.5–E8.5 (HH29-33), the expression is lost from the male (ZZ) gonad and becomes restricted to the active left ovary of ZW (Smith *et al.*, 2008b).

R-spondin-1 (RSPO1) is a member of a small family of secreted growth factors, that also operate through the canonical Wnt signalling pathway. The RSPO proteins are thought to potentially regulate functions mediated by β-catenin, by binding the Wnt co-receptor, LRP6, modulating its availability (Binnerts *et al.*, 2007, Wei *et al.*, 2007). Loss of function mutations within human *RSPO1* result in complete female to male sex reversal (46, XX males) (Parma *et al.*, 2006) and syndromic true hermaphroditism (Tomaselli *et al.*, 2008). Loss of *Rspo1* in mice also results in masculinization of female (XX) gonads due to the absence of WNT4/β-catenin signalling, which result in male-like vascularization and steroidogenesis (Chassot *et al.*, 2008). However, it is not clear if what regulates *RSPO1* expression in the ovary.

In the chicken *RSPO1* is expressed in a sexually dimorphic manner at E4.5 (HH26) at which time its expression is elevated in female (ZW) gonads above the low level observed in male (ZZ) gonads. The mRNA expression level transiently decreases in the female but from E8.5 it is upregulated and becomes strongly female enriched (Smith *et al.*, 2008b). RSPO1 is weakly expressed in the gonadal medulla of both sexes at E6.5 but its expression becomes restricted to the cortex of the left ovary by E12.5 ((Ayers *et al.*, 2013b) Fig. 4A). WNT4 is also expressed within the cortex of the developing ovary at E6.5 and E12.5 along with β-catenin (Fig. 4B). Therefore, RSPO1 way interact with WNT4 to activate β-catenin that is present in these cells.

While both FOXL2 and RSPO1/WNT4 signalling pathways promote ovarian and restrict testis development, it is as yet unclear how these two pathways interact, as mutations in one pathway do not affect the expression of the other (Chassot *et al.*, 2008, Garcia-Ortiz *et al.*, 2009, Ottolenghi *et al.*, 2005). Additionally, these two pathways are expressed in anatomically distinct areas of the developing ovary, as RSPO1 and WNT4/β-catenin are expressed in the cortex (Smith *et al.*, 2008b), while FOXL2 and Aromatase are located in the medulla (Govoroun *et al.*, 2004). And while oestrogen is required to maintain RSPO1 expression in the cortex, it is not clear that there is a direct link between these two pathways, as the decrease in RSPO1 may be due to the loss of pre-follicular cells in the cortex rather than a direct effect on RSPO1 (Smith *et al.*, 2008b). Further research into the mechanisms that regulate ovarian differentiation are needed to further unravel the genes that control sex determination in the female.

As it is still not clear what the female determining gene is in chicken, several laboratories have undertaken large scale screens to identify sexually dimorphic genes in the chicken embryo (Ayers *et al.*, 2013a, Ayers *et al.*, 2015b, Carre *et al.*, 2011, Zhang *et al.*, 2010). Two novel female-enriched candidate genes, *Calpain-5 (CAPN5*) and *G-protein coupled receptor 56* (*GPR56*) were recently identified by RNA sequencing of early chicken gonads (Fig. 5; (Ayers *et al.*, 2015b)).

CAPN5 demonstrated a female biased expression in the gonads of female embryo at stage E6 (HH29; Fig. 5), and was localised to the adrenal gland and the juxta-cortical medulla of female gonads (Ayers *et al.*, 2015b). CAPN5 is an intracellular calcium-dependent cysteine protease that shares homology with the *C. elegans* sex determination gene *tra-3* (Dear *et al.*, 1997, Mugita *et al.*, 1997). Polymorphisms of CAPN5 are associated with of polycystic ovary syndrome in women, suggesting that CAPN5 could play a role in ovarian development (Gonzalez *et al.*, 2006). However, *Capn5* null mice appear to have normal fertility, although gonadal development has not been examined in detail in these mice (Franz *et al.*, 2004).

GPR56 (also known as ADGRG1 - ADhesion G protein-coupled Receptor G1) also displays a strong female bias in the female gonads at E6 (HH29; Fig. 5), and is localised to cells within the cortex of female but not male gonads (Ayers *et al.*, 2015b). *Gpr56* null male mice display reduced fertility due to disruption of the seminiferous tubules, potentially due to loss of basement membrane proteins during testis cord remodelling, which occurs at later stages of testis differentiation in the mouse (Chen *et al.*, 2010). No phenotype has been described for female *Gpr56* null mice and the expression of *Gpr56* has not been described during mouse ovarian development. Equally, the expression of chicken *GPR56* has not been examined in later stages of gonadal differentiation. As such, it is not yet clear whether *GPR56* has a conserved role in gonadal development in chicken and mice, and how this gene could regulate differentiation of the gonad at different stage of development.

Z-linked DMRT1 and the molecular mechanisms underlying testis development

The best candidate avian sex-determining gene under the Z-dosage hypothesis is *DMRT1* (doublesex and mab-3-related transcription factor 1). *DMRT1* encodes a transcription factor that displays sexually dimorphic expression across multiple mammal,

bird and reptile species, where its characterised involvement in testis development suggests it is a conserved component of the vertebrate sex-determining pathway (Smith *et al.*, 1999a). DMRT1 contains a DNA-binding motif termed the "DM domain", which is conserved as evolutionarily far back as the worm (*Caenorhabditis elegans*) gene doublesex and the fly (*Drosophila melanogaster*) gene *mab-3*, both know regulators of male sexual development in their respective species (Matson and Zarkower, 2012, Raymond *et al.*, 2000, Raymond *et al.*, 1998). There are many examples of *DMRT1* and its orthologues being involved in in sex determination and testis differentiation across multiple vertebrate species. In the Medaka fish (*Oryzias latipes*) the master sex determinant is a duplicated copy of *DMRT1*, termed *DMY* (or *DMRT1BY*) because it has translocated onto the Y chromosome (Matsuda *et al.*, 2002, Nanda *et al.*, 2002). Experimentally induced over expression of *DMY* induces male development in genetically female (XX) fish (Matsuda *et al.*, 2007), while two naturally occurring mutants that result in truncated or reduced *DMY* expression cause perturbation of male development in genetically male (XY) fish (Matsuda *et al.*, 2002). More recently, in Zebrafish (*Danio rerio*), DMRT1 has been shown to be necessary for testis development (Webster *et al.*, 2017). In the frog (*Xenopus laevis*), a W-linked dominant negative variant of DMRT1 (DM-W), which lacks the transactivation domain is the likely sex (ovary/female) determining gene (Okada *et al.*, 2009, Yoshimoto *et al.*, 2010, Yoshimoto *et al.*, 2008). Overexpression of DM-W is able to activate the female pathway in ZZ (genetically male) transgenic tadpoles, antagonising the male pathway and autosomal DMRT1 (Yoshimoto *et al.*, 2008). In genetically female (ZW) tadpoles, knockdown of DM-W allows autosomal DMRT1 function and leads to female-to-male sex reversal (Yoshimoto *et al.*, 2010). DMRT1 has also been demonstrated to be both necessary and sufficient to induce male development in two different species of turtles, one that has temperature sex determination (*Trachemys scripta*, Ge *et al.,* (2017)) while the other has genetic sex determination (*Pelodiscus sinensis*, Sun *et al.,* 2017).

In the mouse embryo autosomal DMRT1 expression is restricted to the Sertoli cell linage and germ cells of the developing testis with lower levels present in the developing ovary, concordant with its essential role in post-natal mammalian testis development and function (Matson and Zarkower, 2012, Raymond *et al.*, 1999). Notably, DMRT1 appears to be dis-

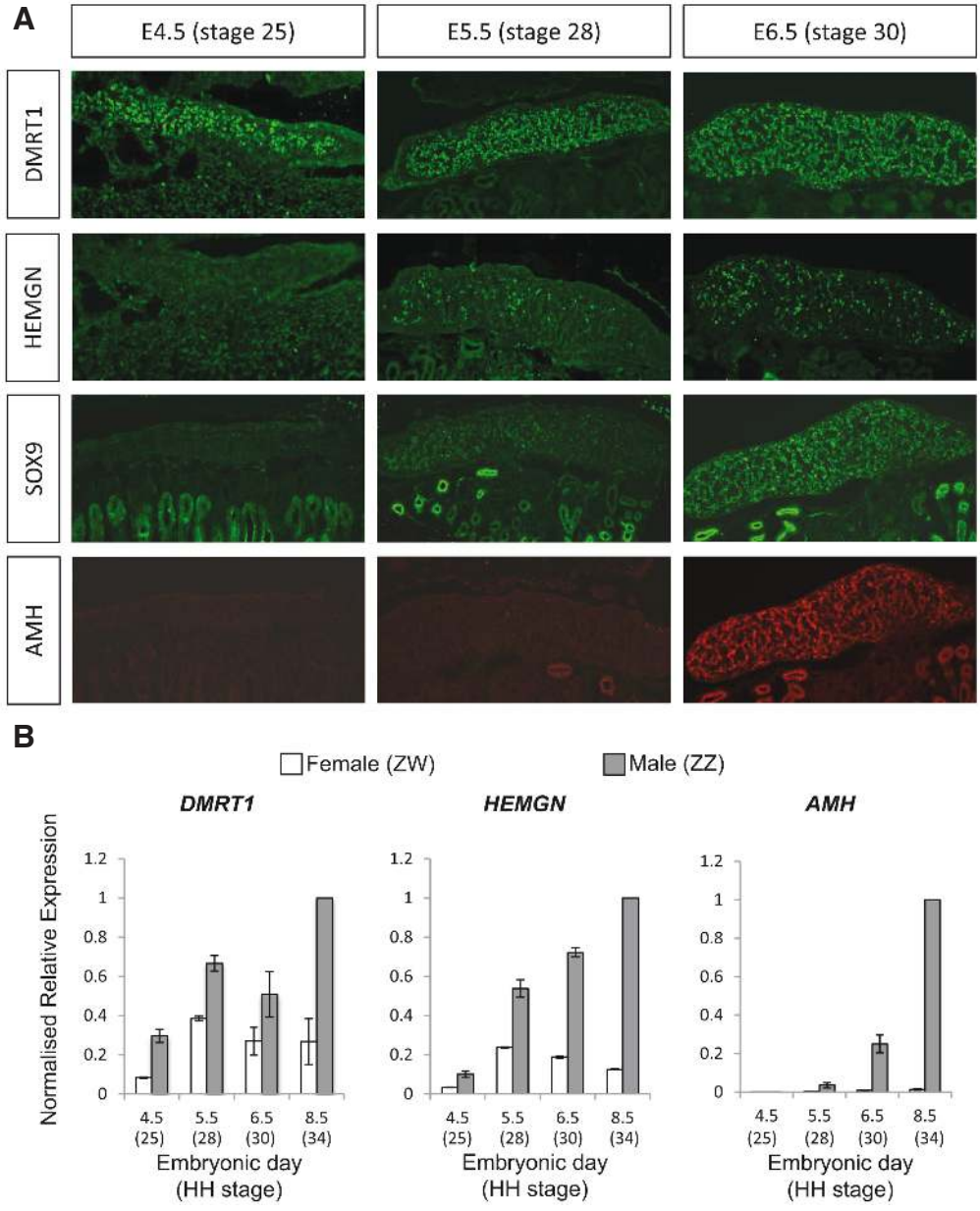

Fig. 6. Chronology of key testis development genes, analysed across several critical stages of chicken sexual differentiation. (A) *Immunofluorescent staining of DMRT1 (green), HEMGN (green), SOX9 (green) and AMH (red) expression in E4.5 (HH25), E5.5 (HH28) and E.6.5 (HH30) male embryonic gonads. DMRT1 expression shows robust expression from E4.5 (HH25) onwards. Both HEMGN and SOX9 were expressed from E5.5 (HH28) onwards, and AMH expression was detectable at E6.5 (HH30), SOX and AMH were co-stains on the same sections.* **(B)** *Quantitative RT-PCR analysis comparing expression of* DMRT1, HEMGN *and* AMH *mRNA in female and male gonads across stages of embryonic development. All transcripts measured were expressed dimorphically with higher levels in the male tissues for each.* DMRT1 *is expressed in a male-biased fashion from E4.5 onwards.* HEMGN *mRNA is initially expressed at a lower level, but becomes male biased at later stages (after* DMRT1 *expression increase).* AMH *was expressed in a sexually dimorphic manner from E6.5.* HPRT *was used as the normalising control and error bars represent SEM, n=3. Reproduced from Lambeth* et al., *(2014), with permissions from Elsevier.*

pensable for embryonic mammalian testis development, with *Dmrt1* null mice displaying a post-natal developmental defect (Raymond *et al.*, 2000). Conditional knockouts of *Dmrt1* in post-natal mice demonstrate that DMRT1 is required in both the Sertoli cells (which otherwise become reprogramed towards granulosa cells) and germ cells (to control the mitosis/meiosis decision) in order to maintain a functional testicular phenotype (Kim *et al.*, 2007, Matson *et al.*, 2010, Matson *et al.*, 2011). Accordingly, mutations or deletions of *DMRT1* in humans are linked with male-to-female sex reversal in 46,XY individuals and an increased risk of germ cell tumours (Bennett *et al.*, 1993, Kanetsky *et al.*, 2011, Veitia *et al.*, 1997).

In the chicken DMRT1 is Z-linked and expressed in the gonads of both genders (germ and somatic cells) and the Müllerian duct, but is more highly expressed in the male gonad (Omotehara *et al.*, 2014). DMRT1 protein is clearly detectable by E4.5 (HH26) in the male (ZZ) embryonic chicken gonad (Fig. 6). Multiple lines of evidence in the chicken support the hypothesis that DMRT1 is the Z-dosage avian sex determinant. Among the first was observational data that *DMRT1* is upregulated in the gonads during female to male sex reversal of ZW embryos using the Aromatase inhibitor fadrozole (Smith *et al.*, 2003). Functional validation has come largely from targeted RNAi mediated knockdown of *DMRT1* using the RCASBP viral vector, with this *in vivo* knockdown approach feminising the left gonad of genetically male (ZZ) chick embryos (Smith *et al.*, 2009a). These gonads displayed female-like morphology, exhibited a loss of SOX9 expression (antagonism of the male pathway) and ectopic expression of Aromatase and FOXL2 (activation of female pathway). Over-expression of DMRT1 in genetically female (ZW) chick embryos has the converse effect on the gonads, inducing the male pathway (activation of SOX9 and AMH), antagonising the female pathway (perturbation of Aromatase expression) and leads to male-like gonad morphology with medullary cells displaying seminiferous cord like organisation (Lambeth *et al.*, 2014). Recent data from the zebra finch (*Taeniopygia guttate*) and emu (*Dromaius novaehollandiae*) have confirmed that the sexually dimorphic expression pattern of DMRT1 observed in the chicken, with increased DMRT1 levels in the male gonads, is conserved within a species from each of the three major bird clades (emu - Palaeognathae; chicken - Galloanserae; zebra finch - Neoaves; Prum *et al.,* (2015)), suggesting that this mechanism is likely conserved across all birds (Hirst *et al.*, 2017a).

All currently available evidence supports the notion that higher expression of Z-linked DMRT1 in the gonads of male (ZZ) chicks acts as the master gonadal sex determinant, triggering a molecular cascade that results in testis formation. Among the downstream targets activated by DMRT1 is SOX9, which is expressed in the developing testes of all vertebrate species examined thus far (Cutting *et al.*, 2013), making it a conserved key molecular cornerstone of male gonadal development and Sertoli cell differentiation (Kent *et al.*, 1996, Morais da Silva *et al.*, 1996, Vidal *et al.*, 2001). SOX9 protein expression is weak at E5.5 (HH28), but readily detected in the nuclei of pre-Sertoli cells in the medulla of the male chicken gonad by E6.5 (HH29), see Fig. 5 and 6A. Due to the time delay between DMRT1 expression (E4.5; HH26) and the peak in detectable SOX9 (E6.5; HH29), DMRT1 is thought to indirectly activate SOX9 expression (Chue and Smith, 2011). The transcription factor Hemogen (HEMGN) is a proposed chicken specific link between DMRT1 and SOX9 (Nakata *et al.*, 2013). It displays male specific expression in the chicken gonad within the appropriate temporal window (Fig. 5 and 6), with HEMGN protein detected in groups of Sertoli cells by E5.5 (HH28). HEMGN overexpression using the RCASBP viral vector in genetically female (ZW) embryos resulted in male-like morphology of the gonad, activation of male markers (SOX9 and DMRT1), coupled with loss of the female associated genes *CYP19A1* and *FOXL2* (Nakata *et al.*, 2013). It is currently unclear if HEMGN can act directly or indirectly to activate SOX9, while the upregulation of DMRT1 (upstream of HEMGN) suggests a positive feedback loop exists, reinforcing DMRT1 expression and the male gonadal differentiation pathway.

In mammals, a positive feedback loop exists between SOX9 and FGF9 (one of its downstream targets in the gonad), where each reinforces the other's expression to establish the Sertoli cell differentiation program while *Fgf9* simultaneously supresses *Wnt4* and the female pathway (Colvin *et al.*, 2001, Kim *et al.*, 2006). In the chicken gonad, however, *FGF9* is expressed at a low level and appears to lack a sexually dimorphic expression pattern, suggesting that this mechanism is not conserved in the chicken (Fig. 5 and Ayers *et al.,* (2015b)). Another SOX9 downstream target characterised in the mouse, Prostaglandin D Synthase (PGDS), also forms a positive feedback loop with SOX9 (Wilhelm *et al.*, 2007). PGDS catalyses the isomerisation of prostaglandin H2 (PGH_2) into prostaglandin D2 (PGD_2), reviewed in Urade and Hayaishi (2000). PGD_2 acts to upregulate SOX9, thereby allowing the PGDS/PGD_2 pathway to act in an amplification loop with SOX9 (Moniot *et al.*, 2009, Wilhelm *et al.*, 2005). *PGDS* mRNA is detected within male (but not female) chicken gonads at E6.5 (HH29), when *SOX9* mRNA can also be detected, suggesting that this pathway is conserved within the chicken (Moniot *et al.*, 2008). Using organ culture of embryonic chicken gonads (and mesonephros) exogenous addition of PGD_2 was shown activate *SOX9* in genetically female (ZW) gonadal explants, but was insufficient to induce *AMH* or full masculinisation of the female gonads in this *in vitro* culture system (Moniot *et al.*, 2008).

AMH expression is upregulated in the male chicken urogenital system with protein readily detected in Sertoli cells along the length of the gonad by E6.5 (HH29), see Fig. 5 and 6. The well characterised role of AMH during embryonic development in mammals is to drive regression of the Müllerian ducts in the male, and its expression is in part controlled by SOX9 during Sertoli cell development (Arango *et al.*, 1999, De Santa Barbara *et al.*, 1998, Josso and Picard, 1986). In the chicken, however, *AMH* mRNA can be detected at E4.5 (HH26), ahead of *SOX9*, and it is also expressed in the gonads of both genders, unlike the mammal where it is only found in the male gonads during embryonic development (Oreal *et al.*, 1998). This is not entirely surprising given that the right Müllerian duct regresses in the chicken, while the left is thought to be protected from AMH by the local actions of oestrogens (Hutson *et al.*, 1982). AMH is required for testis development in several species of fish, some of which lack Müllerian ducts, suggesting that AMH may play roles in gonadal development in addition to Müllerian duct regression in other vertebrate species (Hattori *et al.*, 2012, Kikuchi and Hamaguchi, 2013, Kluver *et al.*, 2007, Morinaga *et al.*, 2007, Nakamura *et al.*, 2012). In the chicken, AMH receptor type-II (AMHR2), which recruits the type-I receptor for intracellular signal transduction, is expressed in the Müllerian ducts and gonads of both genders, but is upregulated in males during gonadal sex differentiation (Cutting *et al.*, 2014). Knockdown of *AMH* in chicken embryos using RNAi doesn't alter the ovarian or

testicular development pathway, however, there was a reduction in the size of the mesonephros and gonads in both genders caused by a loss of proliferation in the cells of these tissues (Lambeth *et al.*, 2015). Overexpression of AMH also had a detrimental effect on the gonads of both genders, forming underdeveloped gonad structures in embryo and the adult (Lambeth *et al.*, 2016a). Female gonads in these animals developed testis-like cord morphology, but like their male counterparts (who had disrupted SOX9 expression), they both lacked Sertoli cells and the capacity for steroidogenesis (Lambeth *et al.*, 2016a). Taken together, these cumulative results demonstrate that while AMH is important for gonadal development in the chicken, it does not have a deterministic role in chicken testis development, but can affect downstream events including steroid production.

Conclusion

The chicken is an excellent model to study the evolution of vertebrate sex determination, as birds combine elements of classic genetic sex determination and share several key genes with mammals, but also retain some features of lower vertebrates such as the central role for oestrogen. The differentiation of the gonads is under the control of several master molecular pathways that control the expression and activity of several important downstream factors, including the male enriched DMRT1, SOX9, PGDS and AMH as well as the female-specific FOXL2-Aromatase and RSPO1/WNT4 pathways.

Until recently, studies into the role of the sex chromosomes, downstream signalling pathways and cell autonomous sex identity in chickens, had relied on either expression analysis or drugs to block the activity of specific genes. However, advances in the genetic manipulation in the chicken now allow functional analysis of candidate genes, through overexpression and knockdown strategies using the RCAS virus (Lambeth *et al.*, 2015, Lambeth *et al.*, 2016a) or electroporation (Ayers *et al.*, 2015a, Hirst *et al.*, 2017c). Furthermore, *in ovo* CRISPR/Cas9 mediated gene targeting has been shown to be a potential tool to achieve loss of function experiments in the chicken (Veron *et al.*, 2015). The integration of data from these gene knockdown and over expression studies, together with analysis of the transcriptome and epigenome will further our understanding of the key regulatory genes involved in sex determination and gonadal differentiation.

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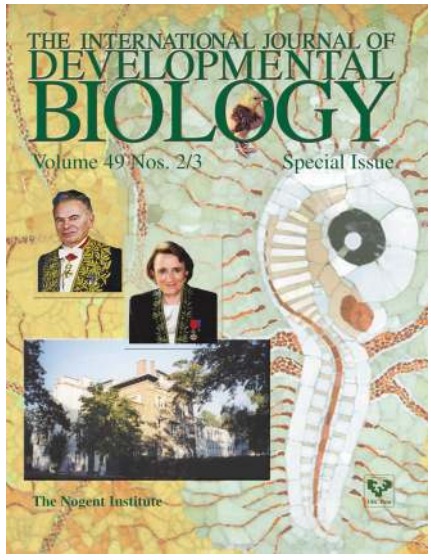

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