Sex determination: insights from the chicken
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Summary
Not all vertebrates share the familiar system of XX:XY sex determination seen in mammals. In the chicken and other birds, sex is determined by a ZZ:ZW sex chromosome system. Gonadal development in the chicken has provided insights into the molecular genetics of vertebrate sex determination and how it has evolved. Such comparative studies show that vertebrate sex-determining pathways comprise both conserved and divergent elements. The chicken embryo resembles lower vertebrates in that estrogens play a central role in gonadal sex differentiation. However, several genes shown to be critical for mammalian sex determination are also expressed in the chicken, but their expression patterns differ, indicating functional plasticity. While the genetic trigger for sex determination in birds remains unknown, some promising candidate genes have recently emerged. The Z-linked gene, \textit{DMRT1}, supports the Z-dosage model of avian sex determination. Two novel W-linked genes, \textit{ASW} and \textit{FET1}, represent candidate female determinants.

Introduction
Sex determination must be a process of great antiquity in animals, being evident in organisms ranging from simple eukaryotes through to mammals. Despite its universal occurrence, sex can be determined by a variety of different mechanisms. Among vertebrates alone, either genetic or environmental factors can be sex-determining (Fig. 1). Mammals and birds exhibit genetic sex determination in which sex is decided by the inheritance of sex chromosomes. Mammals have an XX:XY system, whereby male (XY) is the heterogametic sex. Birds have a ZZ:ZW system, whereby male (XY) is the heterogametic sex. Birds have a ZZ:ZW system, characterised by female heterogamety (ZW). In contrast, temperature-dependent sex determination (TSD) is widespread among lower vertebrates. All crocodilians and many turtles, for example, have TSD. These species lack obvious heteromorphic sex chromosomes and sex is labile, being controlled by egg incubation temperature.\(^{(1)}\)

Sex-determining genes must operate within the embryonic gonads, orchestrating testis or ovary formation. Despite the different sex-determining triggers, the morphology of gonadal sex differentiation among vertebrates is generally conserved. This has led to the long-standing belief that much of the underlying genetic pathway is also likely to be conserved, with essentially only the master switches at the top of the pathway being different in the different vertebrate groups.\(^{(2,3)}\) However, comparative studies on sex determination in the chicken embryo have revealed both conserved and divergent elements. This review will describe insights being gained from the chicken embryo as a model for vertebrate sex determination. The review is framed in an evolutionary context, and will cover both the morphological and genetic aspects of sex determination in the chicken system. It will focus firstly on avian sex chromosomes, followed by the cell biology of gonadal development in the chicken. The role of gonadal hormones will be considered, specifically, the importance of estrogens. Genes implicated in the chicken sex-determining pathway will be described, and current hypotheses for the mechanism of avian sex determination will be evaluated. Finally, experimental strategies to test candidate genes will be outlined. The growing body of genetic data on this species, when combined with its well-documented embryology, identify the chicken embryo as a tractable model for the study of vertebrate sex determination.

The Z and W avian sex chromosomes
Remarkably, the basic mechanism underlying sex determination in birds is still unknown. It may depend upon Z chromosome dosage (two for a male, one for a female) or the W chromosome may carry a dominant ovary-determinant, or both may apply. While birds share genetic sex determination with mammals, their sex chromosomes are not homologous. Comparative gene mapping has clearly shown that the X/Y and Z/W have evolved from different autosomal pairs.\(^{(4)}\) It is not surprising, then, that the testis-determining \textit{SRY} gene, located on the mammalian Y chromosome, is absent in birds. \textit{SRY} appears to be purely a mammalian invention, having evolved...
The chicken Z and W sex chromosomes are shown in Fig. 2. These metacentric chromosomes pair during female meiosis, with a small synaptonemal complex at the tip of the short arms denoting a region of crossing over (the pseudoautosomal region). The Z chromosome is large, accounting for 7.5% of the haploid genome. Several genes have been mapped to the Z, mainly housekeeping genes, but some with more specialised functions. The avian Z chromosome is largely syntenic with human chromosome 9, but it also carries gene blocks that map to human chromosomes 5, 8 and 18. Among those genes mapped to the Z is a candidate male-determining gene, DMRT1 (discussed further below). The smaller W chromosome comprises 1% of the haploid genome and is largely heterochromatic, containing many repeat sequences of the Xho1 and EcoR1 classes (Fig. 2). It is late-replicating and has few bona fide genes. These features make the W analogous to the mammalian Y chromosome. The few genes that have been identified on the W all map to a small euchromatic region near the tip of the short arm. At least three of them (ATP5A, CHD and ASW) have homologues on the Z chromosome. A novel gene, FET1, may be unique to the W and is a possible ovary determinant (discussed below). A candidate W-linked sex-determining gene would clearly need to map outside the pseudoautosomal region, where the W and Z exchange chromosomal material.

**Z dosage or dominant W?**

It was confirmed many years ago that the presence of a Y chromosome triggered male development in mammals regardless of the number of X chromosomes present. Hence, XXY individuals (Klinefelter’s Syndrome) are male, while XO individuals with only one X (Turner’s Syndrome) are female. Unfortunately, definitive evidence for similar sex chromosome aneuploidy in birds is sorely lacking. Early reports of ZZW male chickens or gynandromorphic birds (male on one side of the body, female on the other) were not confirmed by cytology or molecular genetics. One recent study described a gynandromorphic zebra finch, which was ZW female on the left side and ZZ male on the right. This bird showed expression of a candidate W-linked female determinant (ASW) only on the ‘female side’ of the brain. However, since no sex chromosome aneuploidy was involved, this bird does not shed light on the Z dosage versus dominant W hypotheses. It is now thought that sex chromosome aneuploidy in birds may be embryo-lethal, at least in the case of ZO individuals. More recent data from triploid chickens support the Z-dosage hypothesis, antagonised by the W chromosome. Detailed analysis of a ZZW triploid chicken line showed that the birds developed as intersexes. At hatching, these birds had right testes and transient left ovotestes, containing both ovarian and testicular tissue. They were phenotypically female, but the ovarian component of the ovotestis degenerated, and birds became male at sexual maturity. The observations made on these triploid chickens suggest that the W chromosome carries a female determinant, which can be antagonised by the dosage of a Z-linked male-determinant. Regression of ovarian tissue in ZZW triploid chickens implies that the putative W-linked female determinant is not dominant.

Chromosomal evolutionary theory postulates that sex chromosomes become differentiated, following suppression of recombination, to isolate sex-specific genes. In mammals, for example, the highly differentiated Y chromosome carries
the testis determinant, \textit{SRY}, together with genes required for spermatogenesis. These genes are isolated on the \textit{Y}, exempt from recombination with the \textit{X}. Similarly, the chicken \textit{W} chromosome is likely to be differentiated because it carries a female determinant and/or oogenesis genes. The location of oogenesis genes can be inferred from studying the fate of ZZ germ cells in ZW gonads. Experiments with chimeric chicken embryos indicate that, when genetically male (ZZ) blastodermal cells are injected into genetically female (ZW) recipient embryos, some cells enter the ZW ovarian environment as germ cells. These ZZ germ cells enter meiosis and can produce functional oocytes in the absence of a W chromosome.\textsuperscript{(11)} Meiosis is thought to be cell autonomous in birds.\textsuperscript{(12)} Altogether, these data suggest that the W chromosome is not necessary for meiosis, and that oogenesis genes are likely to reside on the \textit{Z} or autosomes. If so, then the sole reason that the \textit{W} chromosome is differentiated from the \textit{Z} may be because it carries a female-determining gene.

\textbf{Gonadal sex differentiation in the chicken embryo}

While sex in higher vertebrates is decided at fertilisation, sex-determining genes become active later during embryogenesis, inducing testis or ovary formation. Gonadal development in the chicken is generally morphologically conserved, providing a good model for other vertebrates. However, it also displays some unique features, which make this system experimentally attractive. As in mammals, the primordial gonads develop from intermediate mesoderm, on the ventromedial surface of the embryonic kidneys (mesonephroi). By day 3.5 of the 21-day embryonic period, the gonads comprise an outer epithelial layer, derived from thickened coelomic epithelium, called the cortex (Fig. 3). Beneath the cortex, the so-called medulla comprises cords of somatic cells separated by loose mesenchyme. The medullary cords appear to derive from proliferating surface (coelomic epithelial) cells.\textsuperscript{(13,14)} Avian primordial germ cells (PGCs) originate in the epiblast and migrate into the gonads via the bloodstream, expressing the conserved \textit{vasa} gene.\textsuperscript{(15)} Gonadal sex differentiation can occur in the absence of PGCs in the chicken embryo, as assessed by histology and hormonal output.\textsuperscript{(16,17)} This is only partially similar to the situation in mammals. Testes can form in the absence of germ cells in the mouse embryo, while meiotic germ cells seem necessary for proper ovary formation.\textsuperscript{(18,19)} The gonads of chicken embryos are “indifferent” or “bipotential” at days 3.5–4.5, being morphologically indistinguishable between the sexes.

The direction of gonadal sex differentiation essentially depends upon which component, the cortex or medulla, proliferates and claims the germ cells (Fig. 3). The onset of differentiation becomes histologically evident at day 6.5.\textsuperscript{(20–22)} This corresponds to Hamburger and Hamilton\textsuperscript{(23)} developmental stage 30. In male (ZZ) embryos, medullary cords thicken, giving rise to seminiferous cords (Fig. 3). This thickening is due to the differentiation and proliferation of Sertoli cells within the cords. As in mammals, this process appears to be the defining morphological step towards testis development. Sertoli cells are supportive cells, protecting and nourishing the germ cells. In mammals, \textit{Sry} is expressed in this cell lineage. ZW (female) chicken embryos show asymmetric gonadal development. Only the left gonad becomes an ovary, while the right gonad grows somewhat and later regresses. In the left gonad, somatic and germ cells proliferate within the cortex, which thickens considerably, while the cords of the medulla become vacuolated, forming so-called lacunae (Fig. 3). Germ cells subsequently enter meiotic prophase (after day 8) and folliculogenesis begins. The granulosa and thecal cells probably derive from somatic cortical cells enclosing the germ cells, or from medullary cells migrating from just beneath the cortex. Although the right gonad forms a vacuolated medulla, similar to that of the left gonad, it fails to form a thickened germ-cell-enriched cortex. This asymmetry of gonadal development
in females makes this a useful developmental system for testing candidate ovary-determining genes. Any genes that are necessary for ovary development might be inactivated in the right gonad, where ovarian development fails to occur.

Several lines of evidence indicate that the presence of the mesonephric kidney is essential for testis differentiation in mammals. Studies in mice show that mesenchymal cells migrate from the mesonephros into the gonad, where they stimulate testis cord organisation and Sertoli cell differentiation. No such mesonephric migration occurs into XX (female) gonads at the same stage of development. This process is clearly important for mammalian testis differentiation, but is it conserved? Merchant-Larios et al. found that microsurgical ablation of the chick mesonephros prior to gonadal development did not prevent normal gonad formation and sexual differentiation, although gonads were reduced in size. However, in these studies, loose mesenchymal cells in the region of the ablated mesonephros could potentially enter the gonad. Other experiments using chick–quail chimeras have shown that formation of the indifferent gonad is independent of the mesonephros, but quail mesonephric cells can enter chick gonads at the time of sexual differentiation. These studies did not examine sex differences in cell migration. It is possible that male-specific cell migration from the mesonephros is a mammalian (or mouse)-specific phenomenon. Another difference between the mouse and chicken models is the differentiation of a prominent coelomic blood vessel in developing mouse testes. This is not seen in the chicken (C.A.S., unpublished data). The cellular mechanisms underlying gonadal sex differentiation among higher vertebrates, therefore, may not be completely conserved. The same conclusion is emerging at the molecular genetic level.

Hormones and avian sex determination: estrogen and AMH
In eutherian mammals, the biosynthesis and secretion of gonadal hormones occurs after sexual differentiation of the gonads. Gonadogenesis itself is essentially resistant to exogenous hormones. Gonadal development in birds is more labile, being susceptible to hormonal manipulation. Sex reversal can be induced experimentally by injecting eggs with estrogens, or by disturbing estrogen production. Such
experiments reveal a critical role for estrogen synthesis in avian sex determination. Synthetic inhibitors of the estrogen-synthesising enzyme, aromatase, can induce permanent female-to-male sex reversal. The left gonad is masculinized (failing to form a normal cortex), while the right gonad becomes a testis. Conversely, ZZ males treated with estradiol become feminised, although this is not permanent. The two terminal enzymes necessary for estrogen synthesis, P-450aromatase and 17βHSD, are expressed only in ZW female gonads at the onset of morphological differentiation (day 6–6.5; stage 29–30) (Fig. 4). These enzymes are expressed in the medullary cords of female gonads. Other enzymes upstream in the steroidogenic pathway are expressed in the medulla of both sexes. Aromatase and 17βHSD are therefore the key sexually dimorphic components. It is logical to suppose that a W-linked female-determining gene may activate both aromatase and 17βHSD expression early in female sex determination. There is no consistent evidence that androgens have an early role in males; testosterone and DHT have no obvious effect when applied to eggs, while androgen receptor is expressed later in gonadal development.

For estradiol to have an effect during gonadal development, its receptor must also be present. Interestingly, estrogen receptor alpha (ERα) is expressed in the gonads of both sexes prior to sexual differentiation in the chicken embryo. This expression is seen primarily in the gonadal cortex. Expression is downregulated in males as development proceeds. In

![Figure 4. Gene expression during gonadal sex differentiation in the chicken embryo. The gonad is morphologically undifferentiated until day 6.5 (stages 29–30; orange box). Circles denote the onset of specific gene expression. Blue circles, male-expressed genes; pink circles, female-expressed genes; yellow circles, genes expressed at similar levels in both sexes. Circle size represents relative expression level compared to the opposite sex. (Data from Refs. 44, 51, 52, 59, 60, 61, 63, 67, 74, 76.)](image-url)
females, expression is also turned off in the right gonad.\(^{32,35}\)

Taken together, these data indicate that estradiol synthesised in the medulla of female gonads mediates female sex determination by stimulating development of the cortex through ER\(\alpha\). Transient expression of ER\(\alpha\) in ZZ embryos explains the susceptibility of males to feminisation by exogenous estradiol. Meanwhile, downregulation of ER\(\alpha\) in the right gonad of ZW females can explain its failure to form a normal ovarian cortex.

Asymmetry of ovarian development in the chicken model may therefore be explained by left–right differences in estrogen action. The mechanisms responsible for establishing asymmetry in ER\(\beta\) expression are completely unknown. ER\(\beta\) has not been detected in embryonic chicken gonads (Q. Hudson, personal communication).

The relative importance of steroidogenesis in sex determination shows an intriguing evolutionary progression among vertebrates. Ascending the phylogenetic tree, from fishes to eutherian mammals, a diminishing role for sex steroids is apparent. Gonadal sex differentiation is widely susceptible to estrogens and androgens in fishes and amphibians,\(^{36,37}\) and susceptible to estrogens in reptiles,\(^{1}\) birds,\(^{29}\) and marsupials.\(^{38}\) In eutherian mammals (mice, humans, etc.), gonadal development during embryogenesis appears resistant to sex steroids and differentiation can proceed in the absence of steroidogenesis.\(^{39,40}\) The common feature shared by non-eutherian vertebrates (including marsupials) is that gonadal sex differentiation usually occurs outside the maternal body. In contrast, gonadal development in eutherian mammals occurs in an environment rich in maternal sex steroids, a risky place if differentiation is estrogen-sensitive. A highly evolved placenta together with intraterine development may have forced eutherians to abandon the use of estrogen as a component of the sex-determining cascade.\(^{41}\)

Female-to-male sex reversal in the chicken embryo can also be achieved by grafting embryonic testes onto ZW (genetic female) embryos prior to sexual differentiation.\(^{42}\) In these cases, both gonads of ZW embryos can develop as testes. It is thought that the responsible factor secreted from the graft is Anti-Müllerian Hormone (AMH). AMH is a glycoprotein hormone produced and secreted by differentiating Sertoli cells of the developing testis, and indeed is considered a marker of Sertoli cell differentiation. AMH mediates regression of the Müllerian ducts in males, which would otherwise form oviducts. The AMH gene is not expressed in female mammalian embryos. Although expressed in early Sertoli cells, AMH in mammals lies downstream within the testis-determining pathway, being expressed after key genes such as SOX9 (see below). Furthermore, targeted mutagenesis shows that AMH is not required for mouse testis determination.\(^{43}\) In the chicken, the onset of gonadal AMH expression begins just prior to sexual differentiation in both sexes, and is consistently higher in males\(^{34,44}\) (Fig. 4). AMH expression in females can be reconciled with the fact that the right Müllerian duct also regresses in ZW embryos. The difference in AMH expression levels between males and females must be important, because additional AMH can fully sex-reverse females, as inferred from the testicular grafting experiments outlined above. Expression of the AMH receptor (AMHR-type II) has not been examined in embryonic chicken gonads.

Could AMH have a more central role in birds, as suggested by the sex-reversing ability of grafted testes? It has an appropriate expression profile, being expressed in gonads just prior to the onset of histological differentiation (Fig. 4). In mammals, as in birds, exogenous AMH can also induce testis (seminiferous) cord formation in female gonads (the so-called freemartin effect). It has been suggested that this is due to the ability of AMH to deplete germ cells, and, since meiotic germ cells seem necessary for ovary formation in mammals,\(^{18}\) this would divert development towards the male pathway. This may also be occurring in the chicken when graft-secreted AMH enters embryonic female gonads. However, as noted above,\(^{16,17}\) gonadal sex differentiation in chicken embryos of both sexes can apparently proceed without germ cells. Therefore, germ cell loss might not be expected to cause testis cord formation in females. It may be possible, then, that AMH has a more central sex-determining (male-determining) role in birds, possibly by repressing aromatase expression. (Studies in mammals have shown that AMH can negatively regulate the aromatase gene, Ref. 45.) This would be a delicate balancing act, given that AMH is also expressed in females, at lower levels, where aromatase repression must not occur. An alternative possibility is that another unknown factor released from testis grafts induces testis differentiation in ZW embryos.

**Conserved genes within the pathway**

Several genes within the mammalian sex-determining cascade have been defined within recent years. Some of these genes have multiple roles, being required for the formation of the indifferent gonad, and for subsequent sex-specific differentiation. Of the genes involved in early gonad formation, most encode transcription factors, such as the orphan nuclear receptor steroidogenic factor-1 (SF1), the Wilm’s Tumour-associated protein, WT-1, and the LIM homeobox protein, LHX9. Null mutations in these genes prevent gonad formation in mouse embryos.\(^{46–48}\) WT1 and SF1 have roles in subsequent testis development, participating in activation of the AMH gene.\(^{49,50}\) Comparisons with the chicken system have shown that the expression profiles for these genes are highly conserved prior to sexual differentiation. Thus, chicken SF1, WT1 and LHX9 are expressed in the indifferent gonads of both sexes\(^{43,51,52}\) (Fig. 4). There is evidence that SF1 and WT1 activate SRY expression in mammals, thereby setting the scene for testis differentiation.\(^{53,54}\) In the absence of SRY, these genes must activate alternative sex determinants in the chicken and other vertebrates. In the case of WT1, differential
Expression of SF1 in the chicken embryo after the onset of sexual differentiation has broadened our understanding of this gene in vertebrate sex determination. In mammals, SF1 is a pervasive activator of steroidogenic enzyme genes, including \textit{aromatase}. In the chicken embryo, SF1 may play a similar role in stimulating \textit{aromatase}, and indeed an SF1 consensus binding site is present in the chicken ovarian \textit{aromatase} promoter region (C.A. Smith, unpublished data). After the onset of gonadal sex differentiation, \textit{SF1} becomes more highly expressed in developing ovaries compared to testes in the chicken embryo.\textsuperscript{(51)} The higher expression in female gonads may be correlated with its greater steroidogenic activity, especially in terms of estrogen synthesis, compared to the testis.\textsuperscript{(28,57)} SF1 may therefore play a role in female-enhanced steroidogenesis, although it alone is unlikely to activate \textit{aromatase}, as males also express SF1 but not \textit{aromatase} (Fig. 4). One possibility is that activation of \textit{aromatase} by SF1 is actively blocked in males, perhaps by the higher AMH levels, as suggested above. In mammals (mouse), SF1 expression declines in the female but is maintained in the male, which is the more steroidogenically active sex. It is interesting to note that the sexually dimorphic pattern of SF1 expression seen in developing chicken gonads is similar to that reported in alligators,\textsuperscript{(58)} the closest living relatives to birds. Thus, SF1 appears to have a conserved role during early gonad formation in vertebrates, but a somewhat divergent role during later sexual differentiation. Other conserved genes also expressed in embryonic chicken gonads include \textit{DAX1}\textsuperscript{(59)} and \textit{FOXL2}\textsuperscript{(60)} (Fig. 4), both initially identified from analysis of mammalian sex-reversing syndromes. The latter is only expressed in female chicken gonads from day 5.5–6, making it a candidate regulator of \textit{aromatase} and \textit{17\betaHSD}.

The first gene found to have a conserved sexually dimorphic expression pattern in vertebrate embryos was \textit{SOX9}. This gene encodes a member of the SOX family of transcription factors, which includes \textit{SRY}. \textit{SOX9} is structurally conserved and is upregulated male-specifically in mammals, birds and reptiles.\textsuperscript{(61,62)} In all of these groups, \textit{SOX9} is expressed in the Sertoli cell lineage of the medullary cords, the first cell type to differentiate in male gonads. In mammals, Sertoli cell formation sets in motion the cascade of cellular events resulting in testis differentiation. \textit{SOX9} has been hailed as the first truly conserved element of the vertebrate sex-determining cascade, controlling Sertoli cell differentiation and responding to different upstream triggers in the different groups. At least one important function has been ascribed to \textit{SOX9} during testis development. It co-operates with SF1 and WT1 to activate expression of \textit{AMH} in mammals.\textsuperscript{(49)} However, studies in the chicken model have shown that even the role of \textit{SOX9} is not completely conserved. In the chicken, the onset of \textit{AMH} expression (at stage 25) precedes that of \textit{SOX9} (at stage 29–30)\textsuperscript{(63)} (Fig. 4). The same relative expression patterns are seen in alligators, the closest living relatives to birds.\textsuperscript{(64)} In the bird–crocodilian lineage, then, \textit{SOX9} cannot initiate \textit{AMH} expression (although it could maintain it). In the chicken embryo, \textit{AMH} is also expressed in the female, but at lower levels. The activator/s of AMH in birds therefore need not be sex-specific, as applies in mammals. It follows that AMH is not necessarily marking Sertoli cell formation in embryonic chicken gonads, being activated in medullary cord cells of both sexes by a common factor. Perhaps a Z-linked gene is responsible, which, being present in two copies in males, induces higher AMH expression in ZZ gonads? In this scenario, \textit{SOX9} could still trigger subsequent Sertoli cell differentiation in males. The gene responsible for activating \textit{SOX9} in mammals has not been identified, although it could be \textit{SRY}. In the absence of \textit{SRY}, \textit{SOX9} must be switched on by another male-specific factor in birds, possibly a Z-linked gene. Since none of the genes discussed so far (\textit{WT1}, \textit{SF1}, \textit{SOX9}, \textit{AMH}) is sex-linked in the chicken, they cannot represent master sex-determinants. The following discussion considers three candidate sex-determining genes that are sex-linked; \textit{DMRT1} (under the Z dosage hypothesis) and \textit{ASW} and \textit{FET1} (under the dominant \textit{W} hypothesis).

**DMRT1 and the Z dosage hypothesis**

According to the Z dosage hypothesis for avian sex determination, the higher dose of a Z-linked gene in ZZ embryos triggers male development, while a lower dose in ZW embryos allows female development. A fundamental requirement of this hypothesis is that the Z-linked candidate gene must escape dosage compensation. Early studies on Z-encoded enzymes indicated higher expression levels in males, resulting in the long-held belief that dosage compensation does not occur in birds. Recent quantitative RT-PCR data, however, show expression equalisation of several Z-linked genes in male and female chicken embryos.\textsuperscript{(65)} Meanwhile, a separate study using fluorescent in situ hybridisation to nascent mRNAs has shown that these Z-linked genes are transcribed from both Z chromosomes in male cells.\textsuperscript{(66)} Therefore, if dosage compensation does occur, it does not involve widespread inactivation of one sex chromosome, as occurs in mammals. One Z-linked gene that appears to escape dosage compensation, however, is \textit{DMRT1} (\textit{Drosophila} Doublesex and \textit{C. elegans} \textit{Mab-3 Related Transcription factor, #1}). \textit{DMRT1} is the best candidate male-determining gene so far identified in birds. It is present on the Z chromosome but has no homologue on the W; a dosage difference therefore exists between the sexes. \textit{DMRT1} is expressed specifically in the gonads of chicken embryos, being more highly expressed in males compared to females prior to and during gonadal sex differentiation\textsuperscript{(67,68)} (Fig. 4). \textit{DMRT1} protein is initially localised within the nuclei of medullary cord cells, where a candidate testis-determinant is expected to operate.\textsuperscript{(51)} Furthermore, in ZW genetic females
that are sex-reversed with an aromatase inhibitor, Z-linked DMRT1 is upregulated in parallel with testis differentiation, despite being present in a single copy.\(^{(31)}\)

DMRT1 expression is conserved among vertebrates, being specific to the urogenital system and upregulated in the developing testis.\(^{(68,69)}\) Perhaps even more fascinating is the observation that the fly and worm homologues, doublesex and mab-3, also have male-specific functions (although not in the gonads). This is the first evidence for conservation of a sex-related gene across different phyla.\(^{(70)}\) However, as for other genes in the sex-determining pathway, the position of DMRT1 may vary among vertebrates. In mouse embryos, Dmrt1 expression does not become sexually dimorphic until after the onset of differentiation,\(^{(67,68)}\) while targeted disruption of the gene does not result in male-to-female sex reversal.\(^{(71)}\) Only postnatal testis dysfunction is observed. A dichotomy may therefore exist between mammals and the egg-laying vertebrates. DMRT1 may represent an ancestral dosage-sensitive testis determinant of birds and lower vertebrates, having been superseded by SRY in mammals. In the mouse, functional redundancy may apply because several other DM domain genes have now been described, some of which are also expressed in the urogenital system.\(^{(72)}\) Such redundancy could also occur in birds. Proof of a role for DMRT1 in the chicken embryo awaits experimental overexpression or knockdown (discussed below).

A novel model for sex determination in the chicken embryo, potentially including DMRT1, involves direct interaction between a W-derived factor and the Z chromosome. Teranishi and colleagues identified a so-called male hypermethylated region (MHM) on the short arm of the chicken Z chromosome, comprising over 200 copies of a 2.2 kb tandem repeat sequence.\(^{(73)}\) This region is hypermethylated and transcriptionally inactive on the two Z chromosomes of males, but is hypomethylated and transcribed on the single Z of females. The MHM region is apparently not translated, but the high molecular weight non-coding RNA coats the Z of females, adjacent to the DMRT1 locus (Fig. 5A). Intriguingly, in ZZZ triploid cells, all three Z chromosomes are hypermethylated and inactive, while, in ZZW triploids, the MHM region is hypomethylated and transcribed from both Z chromosomes.\(^{(73)}\) This clearly implicates the W chromosome in the methylation status of the MHM. The features of the MHM region are reminiscent of the Xist gene in mammals, which produces non-coding RNA that accumulates on the X, causing inactivation and widespread methylation. It is hypothesised that MHM methylation patterns could be involved in sexual differentiation. In ZW embryos, a W-derived factor may induce hypomethylation of the MHM, and the resultant RNA coats the Z, repressing transcription of nearby genes, including DMRT1. In ZZ males, in the absence of the W, the MHM region remains hypermethylated, and inactive, no RNA is made, and no repression occurs (Fig. 5A). This is a very interesting model.

**Figure 5.** Models for avian sex determination, both involving direct interaction between the Z and W sex chromosomes. **A:** Male hypermethylated (MHM) hypothesis. The MHM region in ZZ embryos is transcriptionally silent due to hypermethylation. Neighbouring male-determining genes, such as the candidate gene DMRT1, are free to be transcribed. In ZW embryos, a W-encoded protein (Factor F) induces demethylation of the MHM, resulting in active transcription of high molecular weight non-coding RNA, which accumulates at the site of transcription. Transcription of neighbouring genes, such as DMRT1, is reduced. **B:** Homo/heterodimerisation hypothesis. In ZZ embryos, the ZPKCI gene product operates as a homodimer to stimulate a factor required for testis differentiation. In ZW embryos, the ASW/WPKCI protein heterodimerises with ZPKCI, preventing activation of the testis factor. Alternatively, the heterodimer (or a homodimer, of ASW/WPKCI) may directly activate an ovary factor. Modified from Hori et al.\(^{(74)}\)
and warrants further study. However, if MHM-derived RNA effects DMRT1 transcription in females, its repression must only be partial, because DMRT1 is still expressed in ZW gonads, albeit at lower levels than in males.

**ASW and FET-1: candidate ovary-determining genes on the W chromosome**

Two novel genes have recently been mapped to the euchromatic arm of the chicken W sex chromosome, both showing expression profiles consistent with a role in sex determination. The first of these to be described was ASW (Avian, Sex-specific, W-linked). This gene encodes a protein with some homology to Protein Kinase C Inhibitor, hence its alternative name, WPKCI. However, the predicted protein actually encodes a member of the so-called HINT family of nucleotide hydrolase, but lacking the critical HIT (histidine triad) motif. The HIT motif is required for the hydrolysis of AMP linked to lysine. ASW/ WPKCI is reiterated approximately 40 times on the chicken W chromosome, and is conserved on the W of almost all birds. A single homologue is present on the Z chromosome, called ZPKCI. It does contain the HIT motif, and is therefore predicted to encode a bona fide HINT enzyme. ASW/ WPKCI is strongly expressed in female chicken embryos, especially in the gonads, while ZPKCI shows lower expression in both sexes. HINT proteins act by dimerisation. It has been hypothesised that ASW/WPKCI may play a role in avian sex determination in a dominant negative fashion, by interfering with ZPKCI function via heterodimerisation (Fig. 5B). This idea is appealing from an evolutionary perspective. One can envisage an ancestral state in which ASW/ WPKCI and ZPKCI were entirely homologous, functioning via homodimerisation. Mutation in one allele may have lead to a dominant negative effect on sex determination and the allele evolved into ASW/WPKCI, marking the emergence of the W sex chromosome. It is worthwhile examining this model for avian sex determination in relation to the ZZW triploid chickens described earlier. Despite its extensive duplication on the W, presumably resulting in amplification of its signal, this gene would appear to be insufficient to induce permanent female development in ZZW embryos, which show only transient left ovotestis development.

Some observations undermine the hypothesis that ASW/ WPKCI is ovary-determining in birds. Firstly, its expression is not confined to the gonad in female embryos. It is expressed in several other tissues. Secondly, ASW/ WPKCI appears to be absent in ratites (emus and ostriches). Ratites have homomorphic sex chromosomes, but have GSD like other birds, and presumably rely on the same genetic trigger. (Alternatively, having diverged early in avian evolution, ratites may rely on alternative sex determinants.) In a screen for differentially expressed genes in the chicken gonad, Reed and Sinclair identified what is possibly an even more interesting candidate ovary determinant, FET1 (Female-Expressed Transcript, #1). FET1 is unrelated to ASW, but is located on the euchromatic short arm of the W and does not appear to have a Z homologue. It is expressed almost exclusively in the female urogenital system, with strong expression in the gonads leading up to time of sexual differentiation (day 4.5–6.5; stage 25–30) (Fig. 4). Expression is asymmetric, being significantly higher in the left gonad. As described previously, only the left gonad differentiates into a functional ovary. FET1 has no clear homology to any known genes, but it encodes a predicted 434-residue protein with a putative signal sequence and transmembrane domain. The presence of mammalian orthologues of ASW/WPKCI and FET1 has not been shown. These genes could therefore represent master sex determinants that have evolved only in birds or the bird/reptile lineages, analogous to SRY in mammals. Even if ASW or FET1 are ovary-determining, however, there are still missing links in the pathway leading to aromatase activation. ASW and FET1 are expressed well prior to aromatase, making their direct activation of this gene unlikely without other factors being involved (see Fig. 4).

**Testing candidate sex-determining genes**

DMRT1 on the Z chromosome and ASW and FET1 on the W chromosome represent excellent candidate sex-determining genes in the chicken system. How might these candidates be assessed? The traditional approach in mammals is to use mouse transgenics, either targeted disruption or transgenic overexpression. The reproductive characteristics of the chicken make these approaches difficult and time-consuming. The most-promising strategy for analysing candidate sex-determining genes involves the use of retroviral overexpression in ovo (Fig. 6). Avian retroviral vectors such as RCAS can integrate into the genome and are replication competent. RCAS vectors have been used very successfully to mis-express genes in tissues such as the neural tube and limb. Purified recombinant virus is injected directly into developing embryos, resulting in viral spread and the delivery of exogenous gene expression (Fig. 6). In the case of gonadal development, the objective would be to overexpress DMRT1 in ZW embryos or FET1 in ZZ embryos. Gonadal sex reversal in these instances would provide strong evidence that these genes are sex-determining. However, effective mis-expression of genes using retroviral vectors has not yet been shown in embryonic gonads.

An alternative strategy is to knockdown candidate gene expression using antisense methods (Fig. 6B). This approach uses short antisense molecules to bind and prevent the translation of mRNAs for specific candidate genes. The resulting effect on gonadal development could then be assayed. Morpholino oligonucleotides (MODs) have been used successfully to disrupt the expression of specific genes in the chicken neural tube in ovo, but have not been tested on gonads. Recent refinements to the specificity and delivery of small interfering RNAs (siRNAs) have proven to be very
successful in significantly knocking down the expression of mRNAs in several different vertebrate systems, including developing embryos and isolated organs. RNA interference (RNAi) technology could potentially be applied to the urogenital system in ovo to disturb the expression of genes such as DMRT1, ASW or FET1. The critical issue underlying the efficacy of these methods is achieving sufficient delivery into cells. RNAi has been successfully used to knockdown gene expression in the chick neural tube in ovo, but the gonads have not yet been specifically targeted. Uptake may be enhanced by electroporation. An alternative approach is to treat isolated gonads grown in organ culture with MODs or siRNA (Fig. 6B). Embryonic chicken gonads grown in vitro can differentiate and express sexually dimorphic markers such as SOX9 and aromatase, providing a suitable system for gene knockdown analysis. These experimental approaches will clarify the roles of candidate avian sex-determining genes.

**The chicken embryo as a model for vertebrate sex determination: conclusions**

The chicken embryo provides an excellent model system for studying the developmental biology of vertebrate sex determination. There are some instructive differences between mammals and the chicken model, which are broadening our understanding of sex determination and how it might have evolved. Birds represent an interesting “transitional state” in the evolution of sex determination, sharing GSD and several key genes with the mammals, but retaining some features seen in lower vertebrates, such as the pivotal role of estrogens. Comparisons between birds and mammals show that vertebrate sex-determining pathways are very dynamic, with key genes having multiple roles or having been recruited to different parts of the pathway. An important finding to have emerged from the chicken studies is that sexual dimorphism at the molecular level can occur well prior to sexual dimorphism
at the morphological level. **DMRT1**, **ASW** and **FET1**, for example, all show sexually dimorphic expression as early as day 3.5, well prior to the histological onset of gonadal sex differentiation at day 6.5 (see Fig. 4). Current hypotheses for the mechanism of avian sex determination envisage functional interaction between the W and Z sex chromosomes. If validated, such mechanisms would be different to that in mammals, in which no interaction between SRY (Y-linked) and the X chromosome has been observed (so far). This potential difference is interesting, given that another fundamental sex-related process, dosage compensation, appears to operate very differently in the two groups.

The accessibility of the chicken embryo for experimental manipulation, together with the advent of overexpression and knockdown strategies, make this system potentially advantageous for functional genomic analysis. Until recently, genome research in the chicken has lagged behind that in the mouse and human. However, this situation is being rectified, with chicken BAC libraries (http://www.ark-genomics.org/resources/bacfilters.html) and growing EST resources available (http://www.chick.umist.ac.uk/). Sequencing of the chicken genome is well underway (http://www.ri.bbsrc.ac.uk/chickmap). Integration of this genomic data with the wealth of descriptive embryology will strengthen the position of the chick embryo as an ideal experimental model, not only in the field of sex determination, but for developmental biology in general.

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**References**