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Review
Emu, 2010, **110**, 364–377

ROWLEY REVIEW

Sex determination in birds: a review

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Abstract. In birds, sex determination occurs at fertilisation by the inheritance of sex chromosomes. This review summarises our current understanding of sex determination in birds, with emphasis on the molecular genetics of male versus female development during embryonic life. Recent studies in the Chicken (*Gallus gallus domesticus*) have revealed some remarkable features of avian sex determination, such as the finding that sex appears to be determined autonomously within cells throughout the body, and the demonstration that the key, sex-linked gene, *DMRT1*, is required for testis formation and hence male development. However, despite these recent advances, the mechanism of avian sex determination is still not entirely clear. Understanding sex determination in birds has important implications for the conservation of threatened species, and for the global poultry industry.

Introduction

Birds are well known for their striking sexual dimorphisms, often characterised by gaudy plumage or ornamental feathering in males versus the more drab or cryptic colouration of females. The array of sex-specific features seen in birds and other animals all stem from a key developmental process: sex determination. In its broadest sense, sex determination can be defined as the earliest developmental event whereby sex is established. In birds, and most other animals, sex is determined early in life, at or shortly after fertilisation. In therian mammals (marsupials and 'placentals'), sex is set at fertilisation by the inheritance of the sex chromosomes. Embryos inheriting two X chromosomes develop as females, whereas those inheriting an X and a Y chromosome develop as males. The *SRY* gene, located on the Y chromosome, represents the master sex determinant in higher mammals. In XY embryos, *SRY* initiates formation of testes during embryonic life, whereas formation of ovaries occurs in the absence of *SRY* (reviewed in Capel 1998). However, birds lack *SRY* and have a non-homologous set of sex chromosomes, designated ZZ male and ZW female (Takagi and Sasaki 1974; Griffiths 1991; Coriat *et al*. 1993; McBride *et al*. 1997; Mizuno *et al*. 2002; Stiglec *et al*. 2007). Despite some important advances in the past few years, our understanding of avian sex determination is still incomplete. This review will summarise the current knowledge of avian sex determination at the genetic and developmental levels. Avian sex chromosomes will be described, and the possible mechanism of sex determination will be considered. Sexual differentiation of the embryonic gonads will be outlined and genes implicated in the genetic regulation of this process will be described. Lastly, the implications of understanding sex determination in birds will be discussed.

Avian sex chromosomes

All birds have sex chromosomes and sex is determined at fertilisation by the inheritance of these chromosomes. Sex determination (male *v*. female development) must be regulated at the genetic level by one or more genes located on the sex chromosomes. In birds, male is the homogametic sex, carrying two Z sex chromosomes. Females are heterogametic, inheriting two different sex chromosomes, Z and W. This nomenclature reflects the fact that the sex chromosome system of birds is unrelated to that of mammals. Avian ZZ–ZW and mammalian XX–XY sex chromosomes have evolved from different pairs of autosomes that were present in reptilian ancestors (Fridolfsson *et al*. 1998; Nanda *et al*. 1999; Stiglec *et al*. 2007; Marshall Graves 2008). Birds generally have a high number of chromosomes ($2n > 70$ in most species), comprising several pairs of macrochromosomes, and many small almost indistinguishable microchromosomes (Griffin *et al*. 2007). The sex chromosomes are among the macrochromosome group, whereas the relative size of the Z and W varies among species (Takagi and Sasaki 1974) (shown in Fig. 1). Early karyotyping studies showed that the Z and W are generally very different in size among neognathous (flying) birds. For example, in the Chicken (*Gallus gallus domesticus*), the large Z chromosome comprises 7% of the haploid genome, whereas the smaller W comprises 1.5% (reviewed in Marshall Graves and Shetty 2001). Among the more primitive palaeognathous birds (flightless ratites, such as the Emu (*Dromaius novaehollandiae*), Ostrich (*Struthio camelus*), Southern Cassowary (*Casuarius casuarius*), and the tinamous (Tinamidae)) the Z and W sex chromosomes are usually homomorphic, that is, they appear morphologically very similar or identical (Ogawa *et al*. 1998) (Fig. 1*b*). This is thought to represent a

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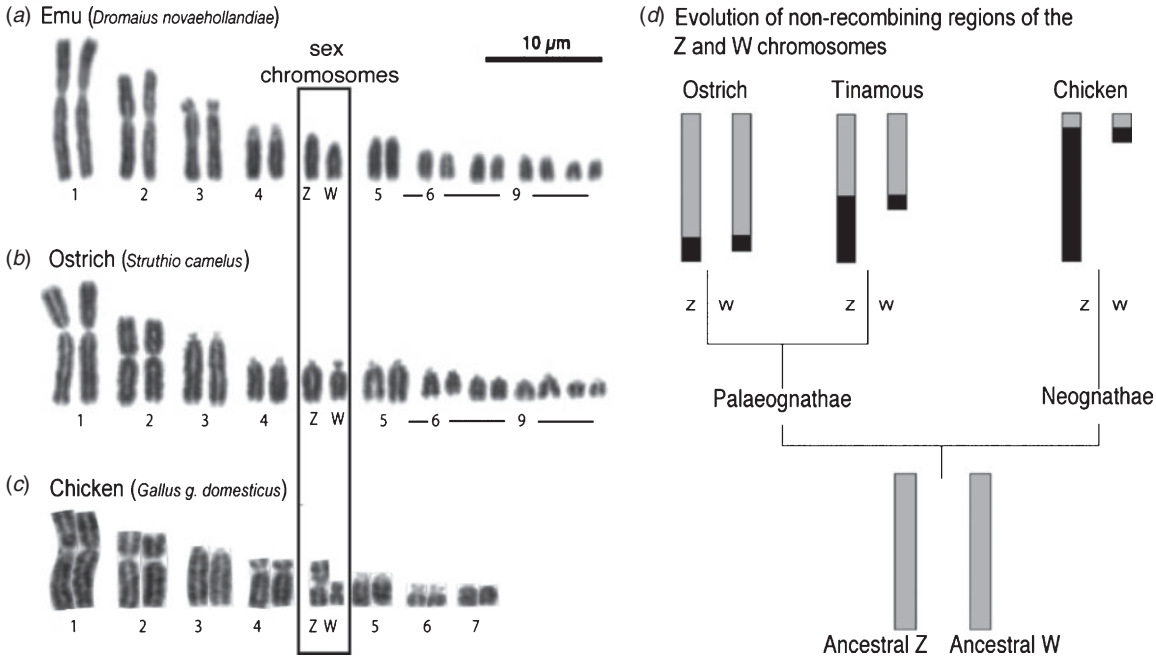

Fig. 1. (*a–c*) Sex chromosomes in ratite (flightless) versus carinate (flying) birds. Giemsa-stained karyotypes of the largest 7–9 chromosomes, with the Z and W sex chromosomes boxed. The sex chromosomes of (*a*) the Emu and (*b*) Ostrich are very similar in size and are considered homomorphic. In the Chicken (*c*) the heteromorphic sex chromosomes are highly differentiated. Modified and reproduced with permission from Nanda *et al*. (1998), and Nishida-Umehara *et al*. (2007). (*d*) Evolution of avian Z versus W sex chromosomes, with recombining regions shown in grey and non-recombining regions in black. The proto-Z and proto-W chromosomes would have undergone recombination during meiosis, as occurs for autosomes. The appearance of a sex-determining gene or genes on one of these chromosomes would favour its isolation in one sex, leading to suppression of recombination around that locus. This would lead to progressive degradation of one sex chromosome owing to its genetic isolation (the W in this case). In the Chicken and other neognathous birds, only a small region of the Z and W still recombine (the PAR), and the W has become much smaller. In contrast, among the ratites, most of the Z and W still recombine in the Ostrich. However, in the tinamous, a non-ratite palaeoganthous bird, the extent of non-recombination is greater. Reproduced from Mank and Ellegren (2007) with permission.

primitive or basal evolutionary state of sex chromosome among birds, with differentiation of the sex chromosomes accompanying the evolution of the neognathous birds, which split from the ratites c. 120 million years ago (van Tuinen and Hedges 2001). (Note, however, that the palaeognathous tinamou has differentiated Z and W chromosomes (Tsuda *et al*. 2007).) It is unclear why the ratite Z and W have differentiated so little over time, unless their sex chromosomes are newly acquired. This seems unlikely given the homology with other birds. Alternatively, other sex-advantage genes may not have accumulated on the ratite Z or W, so that no suppression of recombination, and hence sex chromosome differentiation, has occurred (J. A. Marshall Graves, pers. comm.). Cross-species chromosome painting and comparative gene mapping have shown that the large avian Z sex chromosome is highly conserved among groups, including the ratites (Shetty *et al*. 1999; Nishida-Umehara *et al*. 2007). (Chromosome painting involves fluorescently labelling an entire chromosome of one species and using that to probe to chromosomes of another species. If homologous, the probe will 'paint' the corresponding chromosome.) For example, Nanda and colleagues recently reported that the Z is highly conserved across at least 14 species representing 11 families (Nanda *et al*. 2008). The Z sex chromosome carries ~1000 genes (Bellott *et al*. 2010), most of which are ostensibly unrelated to sex. However, most recently, it has been show that the Chicken Z chromosome, like the mammalian X chromosome, is enriched for testis-expressed genes (Bellott *et al*. 2010). One of these is *DMRT1* (Doublesex and Mab-3 Related Transcription factor, #1), which has a key role in testis development and may represent the master avian testis determinant (Smith *et al*. 2009*a*) (described below).

The Z and W chromosomes of birds have evolved from an ancestral autosomal pair and they are therefore homologous. However, in most birds, the W has lost most of its genes and it now represents a degraded version of the Z. The W is largely composed of repetitive heterochromatic DNA and, unlike the Z, it is not well conserved among different avian groups (Tone *et al*. 1982; Saitoh and Mizuno 1992; Itoh *et al*. 2008). Most ratites exhibit an early stage of sex-chromosome evolution. Little degradation of the W has occurred, its gene content closely resembles that of the Z, and the two chromosomes are of similar size (Fig. 1). When the Chicken Z sex chromosome is fluorescently labelled and hybridised to metaphase spreads of Emu, Ostrich, Southern Cassowary and *Rhea* spp. chromosomes, it paints both the Z and W of all four ratites (Nishida-Umehara *et al*. 2007). This indicates that extensive homology exists between the ratite Z and W, with little evidence of W degradation. In contrast, the Z chromosome of the Chicken and other neognathous birds only paints the Z and not the W. In ratites, the Z and W pair over most of their length during

female meiosis, exchanging genetic material, as with autosomes. However, this region of pairing, the so-called PseudoAutosomal Region (PAR), is small in the Chicken (Fig. 1*d*) (Schmid *et al*. 2005, reviewed in Mank and Ellegren 2007). Taken together, the data indicate that sex chromosomes are poorly differentiated in ratites, but highly differentiated among neognathous birds. Among the latter group, the Z and W sex chromosomes have evidently diverged from each other independently several times during avian evolution (Mank and Ellegren 2007; Itoh *et al*. 2008). Despite the differences among ratites and carinates, all birds have so-called 'genotypic sex determination', whereby the sex chromosomes control development as male or female. The avian sex-determining gene(s) must be present in all birds, including ratites. Given the strong homology of the Z sex chromosome across all groups, and a gene deficit on the degraded W of most birds, it seems unlikely that different sex-determining genes occur in different avian groups, as appears to be the case in bony fishes (Koopman and Loffler 2003; Volff *et al*. 2003). Rather, it is thought that a single sex-determining mechanism applies to all birds, as also in therian mammals.

The molecular mechanism of avian sex determination

The basic mechanism of avian sex determination has remained a mystery for decades. Sex may be determined by Z chromosome dosage (two for a male, one for a female), or it could depend upon a female gene carried on the W sex chromosome. These two possibilities are known as the Z dosage and dominant W hypotheses respectively and they are not necessarily mutually exclusive (Ellegren 2000; Smith and Sinclair 2004; Ellegren *et al*. 2007). Which of these applies? This question could readily be answered by observing the sexual phenotype of sex chromosome aneuploids (birds carrying too few or too many sex chromosomes) (Fig. 2). Hence, a bird with a ZZW genotype that developed as a male despite the presence of the W would support the Z dosage hypothesis, as would a Z0 bird (with only one Z) that was female. Alternatively, a ZZW female bird or a Z0 male bird would support the dominant W hypothesis (Fig. 2). However, such sex chromosome aneuploids have not been definitively documented in birds (Clinton 1998) and it has been suggested that such genotypes may be lethal to embryos (Marshall Graves 2003). Marney Thorne and Bruce Sheldon, working at the CSIRO Animal Production Division, described a line of triploid Chickens, with the genome present in triplicate (3A rather than 2A), including the sex chromosomes (Lin *et al*. 1995; Thorne 1995; Thorne *et al*. 1997). Whereas 3A:ZZZ birds developed as fairly normal males, 3A:ZZW birds developed as intersexes. At hatching, the 3A:ZZW birds had a right testis and a transient left 'ovotestis' with both ovarian and testicular structures (Lin *et al*. 1995). Phenotypically female at hatching, the birds eventually lost the ovarian component of the left gonad and they became male at sexual maturity. This suggests that Z dosage is more important for sex determination in the Chicken, although the W may have some effect. These studies were complicated, however, owing to the fact that the entire genome was present in triplicate, not just the sex chromosomes. More recently, a breeding female Great Reed-Warbler (*Acrocephalus arundinaceus*) was described that was inferred to be a 2A:ZZW, based on Z microsatellite analysis (Arlt *et al*. 2004). Although no karyotyping was carried out to show

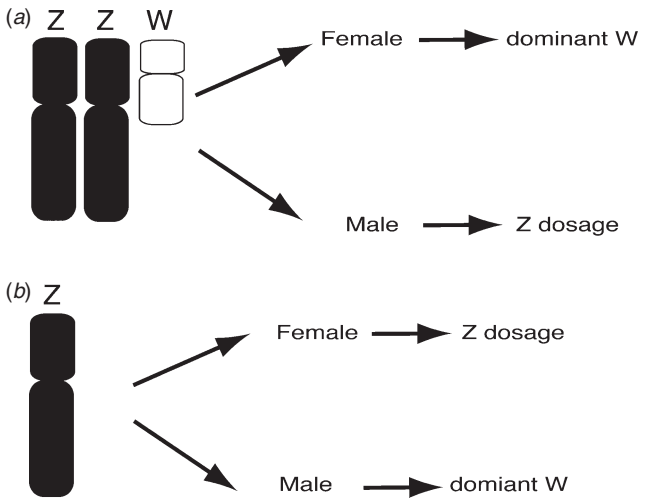

Fig. 2. Sex chromosome aneuploidy and potential sexual phenotypes. (*a*) A bird with a ZZW genotype that was female, despite the presence of two Z sex chromosomes, would support the dominant W hypothesis. However, if the bird was male, despite the presence of the W, this would support the Z dosage model. (*b*) A female bird with a single Z sex chromosome (Z0) would support the Z dosage hypothesis, whereas a Z0 bird with a male phenotype would support the dominant W hypothesis. Such sex chromosome aneuploids have not been definitely identified.

definitively the presence of a W sex chromosome, these data support the dominant W hypothesis over Z dosage. Hence, there is no consistent evidence from these previous studies that clearly supports one hypothesis over the other.

Most recently, Zhao and colleagues, working at the Roslin Institute (Edinburgh, UK), presented a detailed study of three rare but naturally occurring gynandromorphic Chickens (Zhao *et al*. 2010). Gynandromorphs are lateral sex chimeras, male on one side of the body and female on the other (Fig. 3). On the male side, these bizarre birds exhibited a male wattle, large masculine breast muscle, and a large spur-bearing leg. The female side had no male-type wattle, smaller breast muscle and a smaller spurless leg. Zhao and colleagues examined the sex-chromosome composition of the two sides, hoping to find the sex-chromosome aneuploidy that would settle the Z dosage versus dominant W debate. However, cells on the male side contained mainly ZZ sex chromosomes, and those on the female side were predominantly ZW. Internally, the gonads reflected the relative contributions of ZZ and ZW cells: testes were present if most cells were ZZ, ovaries if most were ZW and 'ovotestes' had a mixture of both ZZ and ZW cells. Hence, the gynandromorphs did not shed light on which sex chromosome carries the key sex determinant or determinants. This study, however, does reveal an important new facet of avian sex determination. It suggests that sex determination is cell autonomous in tissues throughout the body, and is not driven exclusively by hormonal signalling. A long-held dogma in vertebrate development is that sex-determining genes control differentiation of the embryonic gonads into testes or ovaries, which then release hormones (testosterone or oestrogens) to masculinse or feminise the rest of the body. There are some notable exceptions in mammals (the formation of pouch and scrotum in wallabies, for example,

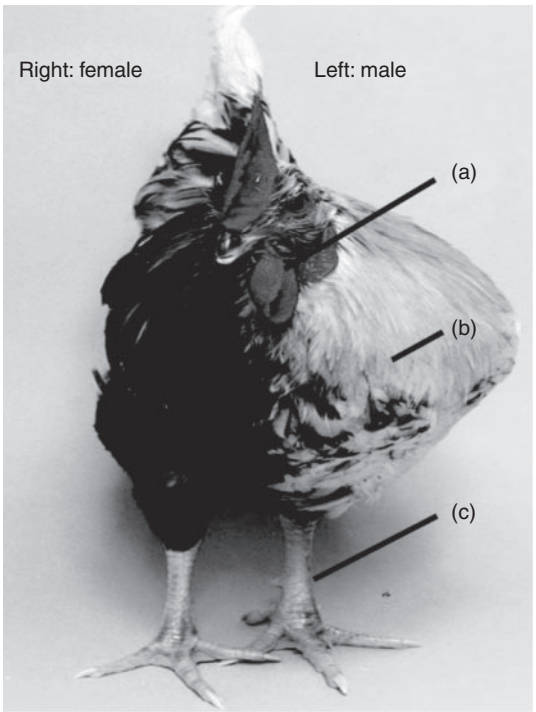

Fig. 3. A gynandromorphic Chicken, with male features on the left and female features on the right. The male side has a wattle (a), enlarged breast muscle (b), and a spur-bearing left leg (c). The female-side lacks these features. Such birds support the notion that sex is established cell autonomously throughout the body. After Zhao *et al*. (2010) with permission.

which precedes gonadal differentiation (reviewed in (Renfree and Short 1988)). The dogma that hormones released from the developing testes or ovaries masculinse or feminise the body could not completely apply to the gynandromorphic Chickens described by Zhao *et al*. (2010); hormones in the circulation would not be restricted to one side of the body. The logical conclusion is that sexual identity is largely controlled by direct genetic affects in each cell, that is that it is cell autonomous (Zhao *et al*. 2010).

Zhao and colleagues also carried out studies on embryonic Chicken gonads experimentally manipulated to carry a mixture of male (ZZ) and female (ZW) cells. These experiments showed that female (ZW) cells would not integrate into male (ZZ) gonads, and vice versa. This again suggested that cells 'know' their sex autonomously and steadfastly retain it (Zhao *et al*. 2010). The authors further postulated that the higher dose of Z-linked genes (the 'Z transcriptome') in ZZ cells would confer 'maleness' in those cells. This idea stems from the finding that there is no chromosome-wide Z inactivation in birds, as occurs for the X chromosome in female mammals to equalise the dosage of X-linked genes between the sexes (Kuroda *et al*. 2001; Melamed and Arnold 2007). Although some genes are dosage compensated, male birds (with two Z chromosomes) have on average twice the level of Z-linked gene expression compared with females (with only one Z) (Ellegren *et al*. 2007; Itoh *et al*. 2007; McQueen and Clinton 2009; Zhang *et al*. 2010). In each cell, the higher level of many Z-linked genes could trigger maleness, or a specific gene could be involved. Other studies also support the idea that cells could have a sexual identity that is at least partly independent of gonadal development. A gynandormorphic Zebra Finch (*Taeniopygia guttata*), for example, had a male-type brain on the left and a female-type brain on the right (Agate *et al*. 2003), whereas sexually dimorphic gene expression has been observed in Chicken embryos well before the onset of gonadal differentiation into ovaries or testes (Scholz *et al*. 2006; Smith *et al*. 2007). Similarly, it has been shown that male Japanese Quails (*Coturnix japonica*) manipulated to carry female brains show female rather than male behaviours, supporting a cell- or tissue-autonomous sexual function, independent of hormonal signalling (Gahr 2003). However, although sex may be established early in avian embryos, this information must be relayed to a genetic switch in the gonad itself that triggers formation of testes or ovaries. Several lines of evidence suggest that this switch is the Z-linked *DMRT1* gene (described below).

Gonadal sex differentiation in avian embryos

A genetic trigger must operate within the gonads of avian embryos to signal formation of testes or ovaries. This trigger must respond to the constitution of the sex chromosome. Much of our understanding of sex determination and gonadal development in birds comes from developmental studies in the Chicken embryo, which has long been a favoured experimental model. Owing to its value as a model in developmental biology, immunology and genetics, and its importance as a major food resource, the genome of the Chicken was one of the first non-mammalian vertebrate genomes to be sequenced (Hillier *et al*. 2004). This has provided a wealth of genomic data that has enhanced our understanding of avian biology, including sex determination and gonadal development.

In birds, the gonads differentiate into testes or ovaries during embryonic life. Gonadal development has been most extensively studied in the Chicken embryo (Clinton 1998; Mizuno *et al*. 2002; Smith and Sinclair 2004). Those few other birds that have been examined at the embryonic stages show a broadly similar pattern of gonadal morphogenesis (Gasc 1978). There is no solid evidence of temperature or other environmental factors having a natural effect upon avian sex determination or gonadal development in birds, as is well documented in reptiles. In the closest living relative to birds, the crocodilians, the temperature of incubation of the egg determines sex (Ferguson and Joanen 1982; Johnston *et al*. 1995; Merchant-Larios *et al*. 2010). However, sex-biased mortality of embryos has been reported in some species, such as the Australian Brush-turkey (*Alectura lathami*), resulting in skewed sex-ratios at hatching (Göth and Booth 2005).

In the Chicken embryo, the embryonic gonads become apparent as thickenings on the ventral medial surface of the embryonic (mesonephric) kidneys by Day 3.5 of incubation (Hamburger-Hamilton stage 19) (Hamburger and Hamilton 1951) At this stage, the paired organs are morphologically indistinguishable between the sexes and are considered 'indifferent' or 'bipotential' (although, as noted above, there are already likely to be sex differences in gene expression). The undifferentiated gonads are composed of an outer epithelial layer, and underlying cords of cells interspersed with loose mesenchyme (the so-called medulla) (C. A. Smith, pers. obs.). Germ

cells (future sperm and ova) originate outside the gonads, in an extra-embryonic region anterior to the head, called the germinal crescent (Fujimoto *et al*. 1976). They subsequently migrate into the embryo and through the developing vascular system to the gonads, where they predominantly populate the outer cortical region. It has been reported that germ cells become asymmetrically distributed in the gonads before sexual differentiation, being more numerous in the left gonad, in both sexes (Vallisneri *et al*. 1990; Zaccanti *et al*. 1990). During sexual differentiation, the gonads develop into either bilateral testes (ZZ) or unilateral ovaries (ZW) (Fig. 4*a*). In genetic males (ZZ) the cords of cells in the medulla condense into seminiferous cords, and the outer

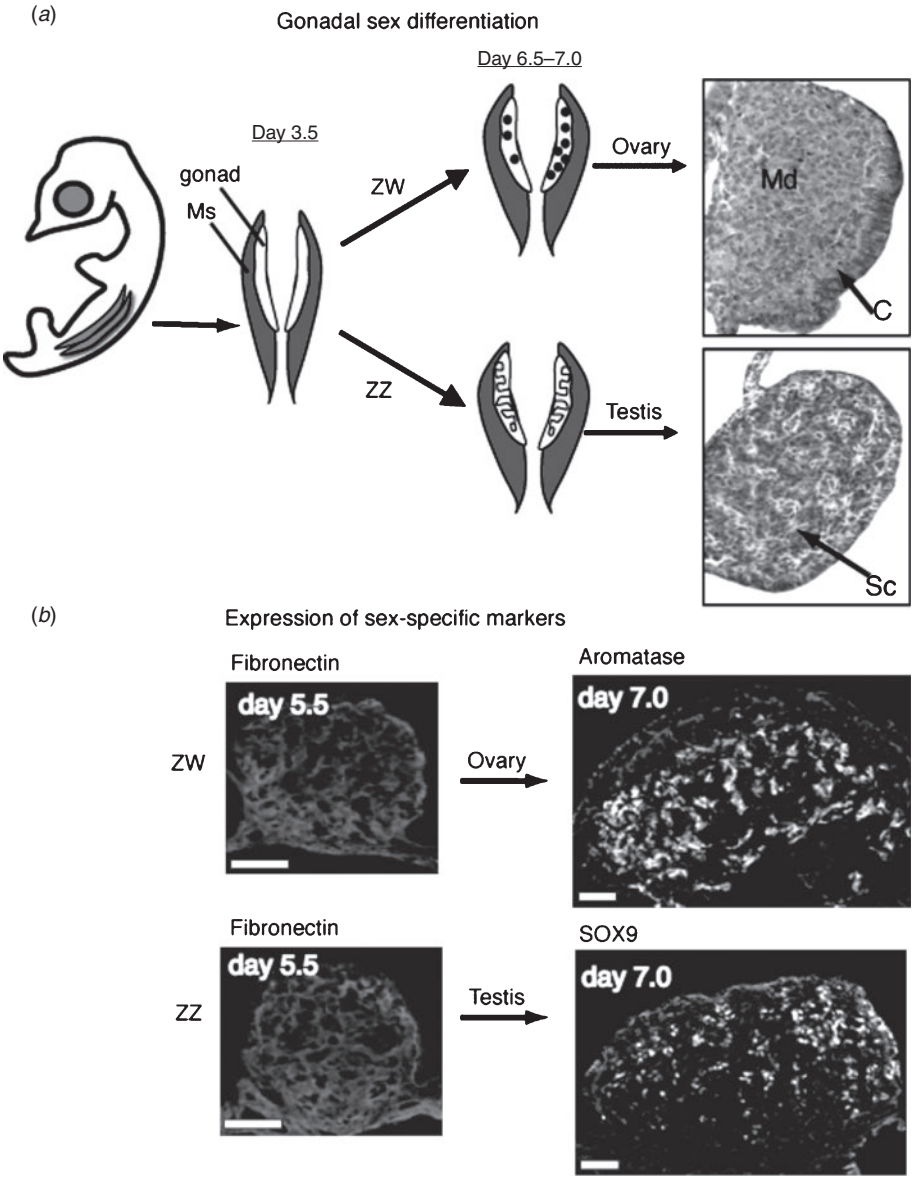

Fig. 4. (*a*) Gonadal sex differentiation in the Chicken embryo. The paired gonads develop on the ventromedial surface of the embryonic (mesonephric) kidneys (Ms). At Day 3.5 (developmental stage 19) the gonads are morphologically identical between the sexes ('indifferent or 'bipotential'). From Day 6.5 (stage 29–30), sexual differentiation begins at the histological level. In ZZ embryos, testis cords condense in the interior part of the gonad (the medulla), marking the onset of the formation of testes. In ZW embryos, the outer zone, the gonadal cortex, proliferates and thickens, marking the initiation of ovarian formation. However, in females, only the left gonad forms a functional ovary, the right gonad regressing. Ms, mesonephric kidney; Sc, seminiferous cords; C, gonadal cortex; Md, medulla. Black circles, germ cells. (*b*) Expression of sex-specific markers in embryonic Chicken gonads. In ZZ and ZW embryos at Day 5.5, fribronectin staining delineates the early gonads. From Day 6.5 to 7.0, the aromatase protein is expressed only in female (ZW) gonads (shown in white). From the same time, *SOX9* protein is only expressed in male gonads (shown in white). Scale bar = 100 μm.

epithelium becomes reduced to a monolayer of squamous epithelium (Fig. 4*a*). Germ cells become enclosed in the cords, where they enter transient mitotic arrest. In female Chicken embryos (ZW), gonadal development is asymmetric. Somatic cells of the left gonadal cortex proliferate, germ cells accumulate in the cortex and enter the early stages of meiosis. The inner medulla becomes fragmented. The right gonad grows initially but never develops a thickened cortex. The medulla becomes fragmented, as occurs in the left gonad, but the right gonad eventually becomes reduced to a small rudiment. This asymmetry has been well characterised at the molecular level, where it has been show to depend upon the homeobox gene, *PITX2*. This gene is preferentially expressed in the left gonad, where it stimulates cell proliferation (Guioli and Lovell-Badge 2007; Ishimaru *et al*. 2008; Rodriguez-Leon *et al*. 2008). It is thought that the regression of the right gonad (and its accompanying oviduct) is necessary because the body cavity of a gravid female can hold only one ovary and duct with developing eggs, and two ovaries and ducts would be energetically expensive for flying. However, this may not be the complete answer, as some bird species have both left and right functional reproductive organs.

At the molecular level, the *SOX9* gene is activated male-specifically at the time of gonadal sex differentiation (from Day 6.5, developmental stage 29–30; Fig. 4*b*) (Kent *et al*. 1996; Morais da Silva *et al*. 1996). *SOX9* encodes a conserved transcription factor, part of the *SOX* family of developmental regulatory genes, of which the mammalian *SRY* gene is the founding member. Expression of the *SOX9* gene is upregulated in the developing testes of amniotic vertebrates (reptiles, birds, mammals) (Kent *et al*. 1996; Smith *et al*. 2005; Shoemaker *et al*. 2007). Evidence from mouse embryos indicates that *SOX9* regulates the differentiation of the Sertoli cell lineage in the developing testis (Kobayashi *et al*. 2005). Sertoli cells are the first type of cell to differentiate in male gonads; they become organised into the characteristic testis cords, they enclose germ cells and they signal the differentiation of hormone-producing Leydig cells. In mammals, current evidence indicates that the Y-linked *Sry* gene activates *SOX9* as one of the first steps in the testis-determining pathway (Sekido and Lovell-Badge 2008). *SRY* is absent in birds, so *SOX9*, which is autosomal, must be directly or indirectly activated by a signal from the sex chromosomes. In female embryonic Chicken gonads (ZW), *SOX9* is never activated. Instead, at the same time (from Day 6, stage 29–30), the oestrogen-synthesising enzyme, aromatase, is activated female-specifically (Fig. 4*b*). This and other lines of evidence indicate that oestrogen plays a key role in ovarian differentiation in avian embryos.

The importance of oestrogen

The steroid hormone, oestrogen, plays a central role in gonadal sex differentiation in the Chicken (Scheib 1983; Vaillant *et al*. 2001; Hudson *et al*. 2005). In embryonic Chicken gonads, all of the steroidogenic enzymes required to produce the oestrogen precursor steroids, testosterone and androstenedione, are present in both sexes (Nakabayashi *et al*. 1998; Nishikimi *et al*. 2000). There is no evidence that androgens influence the early gonadal sex differentiation in birds. The two final-step enzymes required to make 17-β-oestradiol from androgens, P450 aromatase and 17-β-hydroxysteroid dehydrogenase (17-βHSD), are only expressed in female gonads from Day 6 of embryogenesis (the onset of ovarian differentiation; Fig. 4*a*) (Andrews *et al*. 1997; Smith *et al*. 1997; Nakabayashi *et al*. 1998). The importance of oestrogen for female development can be demonstrated by administering a single dose of an aromatase enzyme inhibitor *in ovo*, which results in testis development and female-to-male sex reversal (Elbrecht and Smith 1992; Abinawanto *et al*. 1996; Vaillant *et al*. 2001). As shown in Fig. 5, treatment with fadrozole at Day 3.5 results in greatly reduced aromatase enzyme activity and induces masculinisation of the gonads in ZW (female) embryos. Gonads are stunted, the left fails to form a proper ovary and the right becomes a testis (Fig. 5). Oestrogen receptor α (ER-α), which mediates the action of oestrogen, is expressed in the outer epithelial layer of the left gonads of both male and female embryos (Nakabayashi *et al*. 1998). Hence, the role of oestrogen in females is to promote proliferation of the outer cortical layer of the left gonad. This is the site of germ-cell meiosis in females. Little if any ER-α is expressed in the right gonad, which explains its failure to elaborate a thickened ovarian cortex. The significance of ER-α expression in the left male gonad is unknown, but it explains the ability of exogenously administered oestrogen to feminise the left gonad of ZZ embryos, by promoting cortical proliferation. The trigger for aromatase and 17-βHSD activation in the female gonad is not currently known, but the transcription factor, *FOXL2*, is likely to be involved (Govoroun *et al*. 2004; Hudson *et al*. 2005). In mammals, loss-of-function mutations of *FOXL2* compromise ovarian development (Ottolenghi *et al*. 2005; Pailhoux *et al*. 2005; Garcia-Ortiz *et al*. 2009; Uhlenhaut *et al*. 2009) and *FOXL2* can activate the *Aromatase* promoter region (Pannetier *et al*. 2006).

In contrast to the key role of oestrogen in female development, no equivalent role has been shown for testosterone in early male development. In males, however, Anti-Müllerian Hormone (AMH) may be important for testicular morphogenesis. AMH is synthesised and released by Sertoli cells and is responsible for regression of the Müllerian duct. (This duct otherwise forms the oviduct of females.) AMH is expressed in both sexes (since the right Müllerian duct also regresses in females) but is more highly expressed in males (Oreal *et al*. 1998). Interestingly, if testes at embryonic Day 13 are grafted into early (Day 3) female Chicken embryos, the gonads are masculinised, forming partial or complete testes, with Müllerian duct regression (Maraud *et al*. 1990). It is likely that the masculinising factor involved is AMH. This hormone may therefore have an important role in early testicular development. In contrast, in mammals, where AMH is also male enriched, the hormone has a more downstream role.

Does the W sex chromosome participate in avian sex determination?

The avian W sex chromosome is largely heterochromatic with only a few characterised genes, none of which have an obvious role in sex determination. Yamada and colleagues carried out an extensive expression-based screen of Day 3 and Day 4 Chicken embryos but did not uncover any clear W-linked genes that might regulate gonadal (ovarian) differentiation ((Yamada *et al*. 2004;

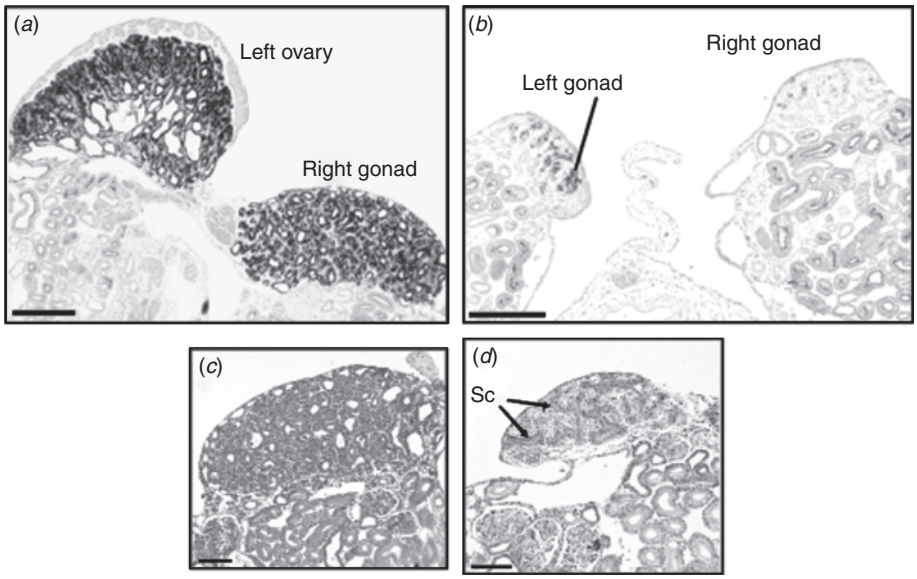

Fig. 5. The effects of aromatase enzyme inhibition on female embryonic Chicken gonads. Gonads at Day 10 are shown. (*a*) Immunohistochemical staining for aromatase enzyme shows expression in the medulla of both the large left ovary and in the smaller regressing right gonad. Note the absence of a gonadal cortex in the right gonad. (*b*) Aromatase staining following administration of the aromatase enzyme inhibitor, fadrozole, on Day 3.5. Both gonads are greatly reduced in size, aromatase staining is reduced in the left gonad and absent in the right gonad. (*c*) Morphology of the right gonad in a female embryo at Day 10. The medulla is riddled with spaces (lacunae) and a cortex is absent. (*d*) Testicular morphology in a right female gonad at Day 10 after aromatase inhibition on Day 3.5. Note the development of male-type seminiferous cords (Sc). Scale bars = 100 µm.

Koyama *et al*. 2007). One W-linked gene that was initially a strong candidate ovary-determining factor is *HINTW* (Histidine triad NucleoTide binding protein) also known as *WPKCI* or *ASW*) (Hori *et al*. 2000; O'Neill *et al*. 2000). *HINTW* is conserved in all carinate birds. In the Chicken embryo, *HINTW* is expressed throughout the body of female (ZW) embryos, including the gonads. *HINTW* encodes an unusual isoform of a nucleotide binding protein of the HIT family. It is unusual because it specifically lacks the AMP hydrolase enzyme activity normally found in these proteins. However, a paralagous gene, called *HINTZ*, is located on the Z sex chromosome. *HINTZ* does encode a bona fide HIT protein, a dimeric enzyme with AMP-lysine hydrolase activity intact. It has been suggested that *HINTW* may function in avian sex determination by inhibiting the (male-promoting) activity of *HINTZ* (Pace and Brenner 2003). *In vitro* enzyme data support a dominant negative influence of *HINTW* on *HINTZ* enzymatic activity (Parks *et al*. 2004). However, the *HINTW* gene is apparently absent in ratites, and overexpression in early Chicken embryos does not induce ovarian development, undermining this gene as a candidate female determinant (Smith *et al*. 2009*b*). At present, therefore, there is little evidence in support of the dominant W hypothesis for avian sex determination.

The DMRT1 *gene and testis development*

The alternative to the dominant W hypothesis for avian sex determination is the Z dosage model, or the Z : Autosome ratio. A large number of genes are carried by the Z chromosome and, in the absence of global dosage compensation mechanism, many Z-linked genes are expressed at a higher level in males compared with females (Itoh *et al*. 2007; Arnold *et al*. 2008). Among these Z-linked genes is a candidate avian sex determinant that is implicated in male development in many animals. The gene is *DMRT1* (Doublesex and Mab-3 Related Transcription factor, # 1). *DMRT1* encodes a zinc finger-like transcription factor, and is structurally related to genes that have a male role in animals as distant as flies (*Drosophila*) and worms (*Caenorhabditis elegans*) (Raymond *et al*. 1998). We and others have found that *DMRT1* has a conserved male upregulated expression pattern in reptile, bird and mouse embryos (Raymond *et al*. 1999; Smith *et al*. 1999; Shan *et al*. 2000). In the Chicken embryo, *DMRT1* is expressed specifically in the urogenital system of both males (ZZ) and females (ZW), but is more highly expressed in males. Mammals with *DMRT1* loss-of-function mutations have impaired testicular development (Raymond *et al*. 2000; Õunap *et al*. 2004). In lower vertebrates, a duplicated and diverged copy of *DMRT1*, called *DMY*/*DMRT1bY*, is the male determinant in the Medaka Fish (*Oryzias latipes*) (Matsuda *et al*. 2002; Nanda *et al*. 2002), whereas a truncated copy, called *DMW*, antagonises *DMRT1* function and participates in ovarian determinant in the African Clawed Frog (*Xenopus laevis*) (Yoshimoto *et al*. 2008, 2010). Hence, this gene and its homologues clearly have a central role in vertebrate gonadal development. *DMRT1* is Z-linked in all birds, including the ratites (Shetty *et al*. 2002). This fact, together with its gonadal expression pattern in developing Chicken embryos, makes *DMRT1* a very strong candidate for an avian testis determinant.

To test the role of *DMRT1* in avian gonadal development, we used an avian retroviral vector to deliver *DMRT1* RNA interfer-

ence molecules into early (Day 0) Chicken embryos. The replication competent viral vector, called RCASBP, spreads from cell to cell following initial infection (Logan and Tabin 1998), delivering short hairpin RNAs (shRNAs) directed against *DMRT1*. RNAi relies on the ability of short antisense RNA molecules to bind to target messenger RNA (mRNA) sequences, marking them for degradation or blocking their translation into proteins, or both. In this way, gene expression is 'knocked down' or silenced. Knockdown of *DMRT1* expression using virally delivered RNAi caused feminisation of genetic males (ZZ) by Day 10 of development (Smith *et al*. 2009*a*). In control females infected with virus carrying non-silencing scrambled control

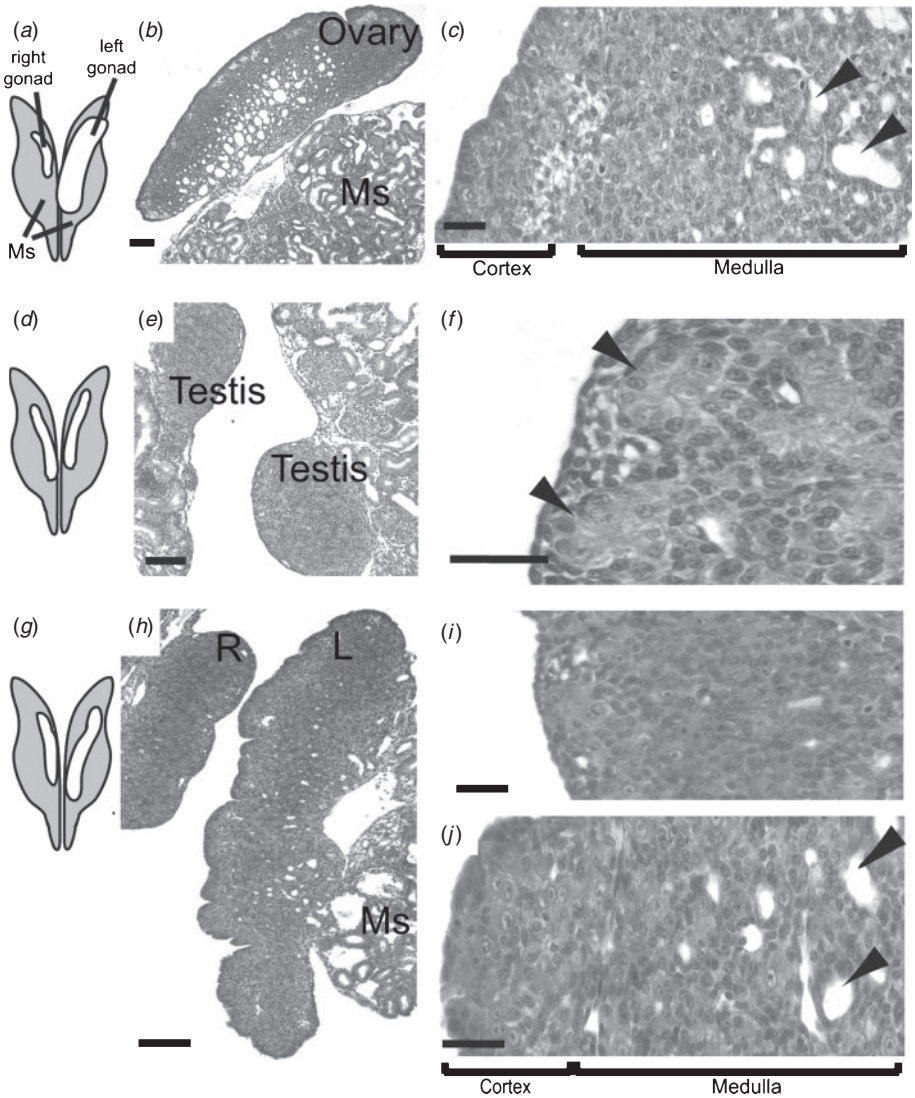

Fig. 6. Knockdown of the Z-linked *DMRT1* gene induces feminisation of male (ZZ) embryonic Chicken gonads. H and E staining of gonads at Day 10 are shown, after embryos were treated on Day 0 with virus-carrying specific short-hairpin RNA against *DMRT1* or scrambled control short-hairpin RNA. (*a*) Control female urogenital system, showing larger left and smaller right gonads. Schematic view. (*b*) Left ovary of a control female. (*c*) High magnification view of a control left ovary, showing thickened outer cortex, and underlying medulla with lacunae (cavities) (arrows). (*d*) Control male urogenital system, showing bilateral testes. Schematic view. (*e*) Histology of control male, showing paired testes. (*f*) Higher magnification of the left testis, showing developed seminiferous cords (arrows). (*g*) Urogenital system of a *DMRT1* knockdown male (ZZ), showing a female-type asymmetry of the gonads. (*h*) Histology of the gonads in a *DMRT1* knockdown male, showing poorly developed right gonad and female-like left gonad. (*i*) High magnification view of right gonad shown in (*h*). Note the lack of organised seminiferous cords. (*j*) Female-like left gonad of *DMRT1* knockdown male, showing thickened outer cortex and underling medulla with lacunae (arrows). Scale bars: in (*b*), (*d*) and (*e*) = 100 μm; in (*c*), (*f*), (*i*) and (*j*) = 25 μm. Ms, mesonephric kidney. After Smith *et al*. (2009*a*) with permission.

shRNA, the gonads developed normally. The right gonad regressed, whereas the left formed a typical ovary, with thickened outer cortex and the inner medulla that was fragmented (Fig. 6*a–c*). In control males, bilateral testes developed, characterised by compact seminiferous cords and a thin outer gonadal (germinal) epithelium (Fig. 6*d–f*). Female embryos subjected to *DMRT1* knockdown appeared normal. However, males subjected to *DMRT1* knockdown exhibited a left ovarian-type gonad and a right testicular or poorly differentiated gonad (Fig. 6*g–i*). The left gonad had a female-type thickened outer cortex and fragmented medulla (Fig. 6*j*). Gene-expression analysis, using quantitative reverse transcription polymerase chain reaction (RT–PCR), confirmed knockdown of *DMRT1* transcripts (Fig. 7*a*). In control males (ZZ) the *DMRT1* protein is expressed in Sertoli cells through the seminiferous cords of the developing testis (Fig. 7*a*). In *DMRT1* knockdown males, little if any *DMRT1* protein is detectable (Fig. 7*b*). At the mRNA level, endogenous *DMRT1* expression is greatly reduced in the knockdown embryos (Fig. 7*c*). In the ZZ *DMRT1* knockdown gonads, expression of the testicular marker, *SOX9*, was significantly reduced, whereas the female marker, *Aromatase*, was ectopically activated (Fig. 7*d*, *e*). Taken together, these data show that the Z-linked gene, *DMRT1*, is required for proper male development in the Chicken embryo.

The *DMRT1* knockdown experiments support the Z dosage model of avian sex determination, whereby *DMRT1* dosage directs testicular versus ovarian development (higher in males). Is *DMRT1* the master avian sex-determining switch gene? It is expressed at the gonads before and during sexual differentiation, it is conserved on the Z in all birds, and knockdown induces male-

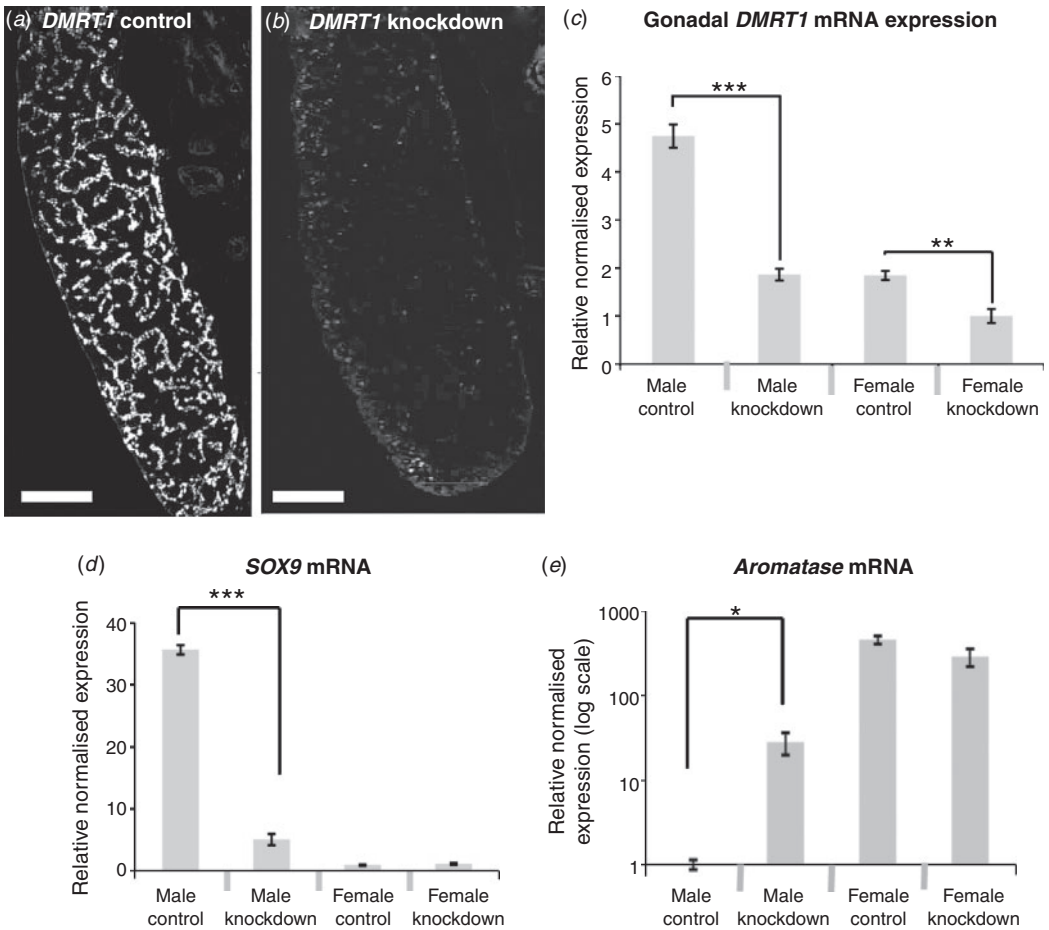

Fig. 7. Changes in gene expression following *DMRT1* knockdown in embryonic Chicken gonads at Day 10. (*a*) Control male gonad (testis) showing *DMRT1* protein expression throughout the cords of the testis. Immunofluorescence; scale bar = 100 µm. (*b*) Male gonad following *DMRT1* knockdown, showing little if any *DMRT1* protein. Scale bar = 100 µm. (*c*) Decline in *DMRT1* mRNA expression in *DMRT1* knockdown versus control male and female gonads. Bars show mean ± s.e.m.; differences between means tested using *t*-tests, with ***, $P < 0.001$; **, $P < 0.01$. (*d*) Downregulation of *SOX9* mRNA in control and *DMRT1* knockdown gonads. In controls, *SOX9* is highly expressed in males, and not in females. Bars show mean ± s.e.m.; differences between means tested using *t*-tests, with ***, $P < 0.001$. (*e*) Activation of *Aromatase* mRNA expression in male *DMRT1* knockdown gonads (log scale). In controls, aromatase is highly expressed in females but not in males. Bars show mean ± s.e.m.; differences between means tested using *t*-tests, with *, $P < 0.01$. Modified from Smith *et al.* (2009*a*) with permission.

to-female sex reversal in the Chicken embryo. It is theoretically possible that another Z-linked gene lies upstream of *DMRT1* in the testis pathway, although this seems unlikely. In addition, in tissues outside the gonads, the gynandromorphic Chicken study (Zhao *et al*. 2010) suggests that other Z-linked genes may operate to regulate sex outside the gonads, because *DMRT1* is not expressed outside the urogenital system. *DMRT1* is required for testis development, but is unlikely to be the master sex-determining switch in other tissues or the embryo as a whole, since cells appear to know their sex before formation of the gonads (Zhao *et al*. 2010). Hence, there are still several questions surrounding avian sex determination, and the picture is not yet complete. The nature of the cell autonomous sex-determining switch outside the gonads remains to be defined. Is it controlled by several Z-linked genes, or by different genes in different tissues, or by an as yet undefined W-linked gene?

Avian sex chromosomes and sex-determining genes: applications to wild and domesticated birds

The analysis of avian sex chromosomes and sex-determining genes has important implications for both wild and domesticated birds. First, DNA sequences derived from the sex chromosomes provide valuable tools for the molecular sexing of birds. This allows the sexing of fledgling or immature birds, and mature birds that are not sexually dimorphic, and provides critical information for the breeding of rare or endangered species. Second, by genetically manipulating key sex-determining genes, the sex of commercially produced birds can potentially be altered. This is of value in the poultry industry, for example, where females are required for laying, whereas males are required for meat production.

Birds can be sexed at the molecular level by utilising sex-linked DNA sequences. For example, repetitive sequences of the *Xho*I family occupy ~70% of the W sex chromosome of the Chicken (Kodama *et al*. 1987; Klein and Ellendorff 2000). Amplification of these elements by polymerase chain reaction (PCR) is now routinely used to sex Chicken embryos (Clinton *et al*. 2001). Females show a DNA band on an agarose gel whereas males do not. However, such repetitive elements are rapidly evolving and the W-linked *Xho*I repeat is absent outside the Galliformes (which includes the Chicken, turkeys, pheasants and other gamefowl). Alternative W-linked sequences that are more widely conserved have been utilised to sex a variety of avian species, from many different families. For example, the conserved W-linked gene, *CHD-W* (ChromoHelicase DNA-binding protein-W linked) has a homologue on the Z (*CHD-Z*) but the two sequences can be distinguished by different sized introns, or by the presence of unique restriction enzyme sites (Griffiths *et al*. 1996; Chang *et al*. 2008). This has allowed the use of the sex-linked *CHD* gene for the molecular sexing of several different birds, including rare species (Ellegren 1996; Griffiths *et al*. 1998; Wang *et al*. 2007). The last remaining Spix's Macaw (*Cyanopsitta spixii*) was sexed in this way (Griffiths and Tlwarl 1995). The *CHD-W* and *CHD-Z* genes can be readily distinguished in all birds, with the exception of the ratites (Emu, Ostrich, Southern Cassowary, rhea, kiwi (*Apteryx* spp.) and tinamous). This is consistent with the near-complete homology of the Z and W sex chromosomes in most members of this group (Ogawa *et al*. 1998; Nishida-Umehara *et al*. 2007). However, even among this group, molecular sexing has been successfully carried out, using an unrelated W-linked (female-specific) sequence (Huynen *et al*. 2002). Hence, all birds can now be sexed at the molecular level, enhancing studies on avian reproductive biology, ecology, breeding and conservation.

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Manuscript received 28 April 2010, accepted 5 August 2010