Gonadal Asymmetry and Sex Determination in Birds

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Vertebrates display a superficial bilateral symmetry, a basic body plan in which the left and right sides form mirror images across the midline, but internally most organs develop and locate asymmetrically [Wolpert, 2005]. These include the heart, spleen and stomach, and also bilateral organs such as lungs and kidneys which differ in appearance or position on the right and left sides. Laterisation also affects the structure and the function of the brain hemispheres, generating a spectrum of asymmetric behaviours specific to different vertebrates (e.g. handedness in humans) [Vandenberg and Levin, 2013]. To add to the complexity, some tissues which develop symmetrically, such as the somites, do so despite the expression of left:right (L:R) determinants, because the asymmetry is overridden by retinoic acid (RA) signalling [Vermot and Pourquie, 2005; Duester, 2007; Vilhais-Neto et al., 2010].

In recent years, enormous progress has been made in understanding the molecular and developmental basis of avian sex determination, and discuss the possible biological reasons why many birds have adopted a single-ovary system.
Morphology

In chick embryos, as in mammalian embryos, the gonads arise as a thickening of the coelomic epithelium which forms a ridge running in an anterior/posterior orientation on the ventral surface of each mesonephros (primitive kidney). Gonadogenesis begins at around 72 h of development (Hamburger Hamilton (HH) developmental stage 23) [Hamburger and Hamilton, 1951], but the genital ridges are not macroscopically evident until HH26 when both are approximately 1.5 mm in length and 0.1 mm wide (fig. 1a) [Swift, 1915; Romanoff, 1960; Carlon and Stahl, 1985]. As development proceeds, the morphological appearance of the gonads is initially similar in males and females (HH28), but by HH36, the gonads in males and females are distinctly different. The right and left testes become tubular structures, approximately 3 mm long and 0.5 mm wide, and while the right female gonad is similar in appearance but slightly smaller than the testes in males, the left female gonad has acquired a broader, flatter appearance and is markedly larger at approximately 3.2 mm long and 0.8 mm wide (fig. 1b–h). Throughout embryonic development, male and female gonads continue to increase in size and immediately prior to hatching both testes are approximately 5 mm long and 1.5 mm wide, while in the female the right gonad is around 2.6 mm by 0.5 mm and the left, which is clearly an ovary, is 8 mm by 1.5 mm. The physical growth of the gonads between HH36 and HH44 is reflected by increases in wet weight and RNA content (fig. 1i–l).

In the initial stages of gonadogenesis, the epithelial ridge is composed of columnar cells that overlie tissue that lacks the typical nephron structure characteristic of the underlying mesonephros and that is instead composed of clusters of epithelial-like cells embedded in mesenchyme [Carlon and Stahl, 1985]. By HH25, the ‘genital ridges’ protrude into the coelomic cavity as distinct organs and comprise a pseudo-stratified columnar epithelium (the germinal epithelium) covering a central core (the medulla) organised into epithelial cords. The origin of these so-called primitive sex cords, which are also apparent in some mammals such as humans, but not the mouse, is still unclear. Some studies indicate the germinal epithelium as a source, while others suggest that they are of mesonephric origin [Stahl and Carlon, 1973; Merchant-Larios et al., 1984; Rodemer-Lenz, 1989; Sekido and Lovell-Badge, 2007]. At this point, both male and female gonads already display a L:R morphological asymmetry, with the epithelial layer on the left gonad thicker than that on the right [Carlon and Stahl, 1985] (fig. 2). This asymmetric feature is maintained in both sexes at the time of gonadal sex determination (thought to be around HH27–28). By HH29, the epithelium surrounding the right gonad flattens and adopts a more squamous-like appearance, further emphasising the L:R asymmetry in both sexes [Carlon and Stahl, 1985]. As gonadal sex differentiation progresses, the morphological differences between left and right gonads in the female become more pronounced, while the asymmetry between male gonads diminishes (fig. 1c–h, 2). By HH36, both left and right testes have a similar organisation: a thin flat simple epithelium overlaying a core organised into well-defined branched tubular structures. These branched structures are designated sex cords or testis cords and comprise germ cells and differentiating Sertoli cells encased within
a basement membrane (fig. 3b–d). In contrast, the overall structure of left and right ovaries is clearly different and these differences are largely confined to the outer epithelial layer (fig. 3a). While the right ovary, like the testes, is surrounded by a thin flat simple epithelial layer, the left ovary is enclosed in a thick sheath of stratified epithelial-like cells, known as the cortex (fig. 2, 3a) [Romanoff, 1960]. The medulla has a similar organisation in both ovaries with irregular disorganised cords of epithelial-like cells and vesicular structures known as lacunae. By HH39, most of the cells of the cortex are organised into cord structures around groups of germ cells and these ‘secondary’ cords extend into the medullary region [Gonzalez-Moran, 2011].
The L:R asymmetry associated with the embryonic gonads is not confined to the somatic component. There is also a clear L:R asymmetry in the distribution of the germ cells (fig. 4). As early as HH15, the number of primordial germ cells (PGC) in the intermediate mesoderm in the area beneath the forming ridges, was found to be consistently and significantly higher on the left than on the right in both sexes with an approximately 3:2 L:R split by HH17 [Nakamura et al., 2007]. This trend persists and accentuates during the colonisation of the genital ridge so that in both sexes, the left gonad contains more germ cells than the right (fig. 4a). This asymmetry has been observed in the chick and other birds (e.g. quail and duck) [Van Limborgh, 1968; Didier and Fargeix, 1976; Bergeaud et al., 1977; Dubois and Cuminge, 1978; Nakamura et al., 2007; Intarapat and Stern, 2013]. There is evidence that in the chick, as in the mouse, PGC colonisation of the gonads is driven by chemoattractants secreted by the gonadal mesoderm [Molyneaux et al., 2003; Stebler et al., 2004]. So it is possible that the unequal L:R distribution of germ cells is simply due to a L:R asymmetry in the level of gonadal chemokines – a hypothesis first suggested by very early studies on germ cell migration [Witschi, 1935; Baillie et al., 1966; Dubois, 1968]. Interestingly, it has been reported that between HH22 and HH26 in chick, well before gonadal sex determination, this asymmetry is more pronounced in females than in males with an approximately 4:1 and 2:1 L:R split, respectively, indicating that at least some sex-specific differences are present before the accepted time of sex determination [Van Limborgh, 1968]. Moreover, unlike the situation in mammals, the germ cells are not initially distributed throughout the genital ridges, but are mostly localised close to the epithelium in the left and in the right gonad in both females and males (around 70–85%) [Van Limborgh, 1968]. However, by HH30 this situation persists only in the left female gonad, while the germ cells become randomly distributed throughout the right female gonad and throughout both left and right male gonads (fig. 4a).

This particular asymmetry is maintained into the later stages of ovary development (HH44) so that in the left ovary, the germ cells are found predominately in the cortex, and in the right ovary, a smaller number of germ cells appear to be randomly distributed throughout the core region. In the male, similar numbers of germ cells are located in the sex cords of both right and left testes (fig. 4b).

A number of studies have followed the fate of germ cells in the period immediately prior to hatching. It has been proposed that the germ cells of the right embryonic ovary are lost by cell death or cell abandonment based on the identification of apoptotic germ cells and of germ cells within the lacunae [Ukeshima and Fujimoto, 1991; Ukeshima, 1994, 1996]. However, a more recent study [Gonzalez-Moran, 2011] reports that the total number of germ cells in the right ovary increases up to the point of hatch, suggesting that any loss is compensated for by germ cell proliferation. The same study found that the number of germ cells in the medulla of the left ovary decreases after HH38–39 indicating a progressive elimination during the second half of embryogenesis [Gonzalez-Moran, 2011].
is also noteworthy that, by HH41–42, most of the cortical germ cells of the left ovary have entered meiosis [Hughes, 1963; Ukeshima and Fujimoto, 1991; Smith et al., 2008a; Yu et al., 2013]. At this time, medullary germ cells of both female gonads do not possess the typical morphology of meiotic cells, nor do they express typical leptotene proteins, such as SCP3 [Ukeshima and Fujimoto, 1991; Smith et al., 2008a], suggesting that germ cells in the medulla either never enter meiosis, or are delayed in doing so. By 4 weeks post-hatch, the medulla in both left and right female gonads has lost all germ cells and is organised into large compacted cords with much reduced lacunar channels [Gonzalez-Moran, 2011].

The right female gonad does not develop into a functional ovarian structure in the adult and is considered to eventually atrophy. However, the removal of the left ovary in the young chick results in the differentiation of the right gonad into a testis-like organ [Groenendijk-Huijbers, 1965, 1967], indicating that the right gonad is not completely lost, and there is evidence suggesting that the right ovary can maintain a steroidogenic function [Narbaitz and Kolodny, 1964; Samar et al., 1983].
In contrast, the left ovary becomes a fully functional reproductive organ similar in structure to the ovary of all other vertebrates: a central medulla mainly composed of stromal-vascular tissue and a cortex containing meiotic oocytes surrounded by granulosa cells and steroidogenic cells [DeFalco and Capel, 2009].

Molecular Asymmetry

Analysis of cell growth at HH27–29 showed that the proliferation rate in the epithelium of the left gonad is higher than that found in the epithelium of the right gonad in both male and female embryos, while the prolif-
eration rate in the right and left medulla of both sexes was similar [Ishimaru et al., 2008]. It is possible that this differential proliferation is due, at least partially, to steroidogenic factor 1 (SF1). This transcription factor is expressed in the medulla of left and right genital ridges, but only in the left epithelium. The finding that SF1 up-regulates the expression of the cell cycle regulator cyclin D1 (CD1) and increases cell proliferation in the epithelium led to the proposal that SF1 may directly activate the CD1 promoter, perhaps through interaction with β-catenin [Ishimaru et al., 2008] via a similar mechanism to that seen with CD1 activation by the SF1 homologous gene LRH1 [Botrugno et al., 2004]. However, CD1 activation alone cannot account for ovarian asymmetry: while CD1 activation in the right epithelium led to an increase in overall gonadal size, it was not sufficient to induce cortical differentiation [Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

Cells of the gonad epithelium have also been shown to display an asymmetry in relation to the orientation of the plane of division [Rodriguez-Leon et al., 2008]. In a typical epithelial monolayer, cell division can be either perpendicular or parallel to the epithelial plane. With the former, both cells remain in the epithelial layer and this is described as a symmetrical division, while the latter may result in 1 daughter cell leaving the monolayer leading to an asymmetric division [Betschinger and Knoblich, 2004; Woolner and Papalopulu, 2012]. At HH27, the percentage of potential asymmetric cell divisions in the epithelium of the left gonad is almost twice that found in the epithelium of the right gonad [Rodriguez-Leon et al., 2008]. While the significance of this finding is not certain, it is known that symmetrical divisions result in epithelial growth, whereas asymmetric divisions can generate stratified epithelia, and/or produce new cell types [Baena-Lopez et al., 2005; Woolner and Papalopulu, 2012]. It is possible that the combination of a higher proliferation rate and a bias in favour of asymmetric cell division on the left side may contribute to the patterning of the cortex.

Not surprisingly given the morphological differences between the left and right epithelia, several cell adhesion and extracellular matrix components have also been shown to display L:R asymmetry [Guioli and Lovell-Badge, 2007; Rodriguez-Leon et al., 2008]. These include N-cadherin and cytoplasmic β-catenin which show distinct subcellular localisation in left and right epithelia from HH28 [Rodriguez-Leon et al., 2008]. Moreover, the basal lamina beneath the epithelium acquires a different structure on the left compared to the right by HH28. Indeed it appears more discontinuous and enriched in fibronectin (fig. 2) [Guioli and Lovell-Badge, 2007]. Adhesion properties are obviously important determinants in various aspects of epithelial morphogenesis, including spindle orientation, cell shape and cell migration, and are therefore potential players in the fate of left and right epithelia [Nelson, 2009; Cai and Mostov, 2012].

In the female chick embryo, both the left and the right gonadal medulla synthesise estrogen from HH29–30 onwards, as assessed by expression of P450 aromatase (the enzyme that catalyses the conversion of androgens into estrogens) (fig. 5a) [Andrews et al., 1997; Nakabayashi et
This steroid hormone modulates gene transcription and activates cell signalling, mostly via binding to estrogen receptors (ERs) [Marino et al., 2006]. The only ER found to be expressed in the embryonic gonad is ERα, and this receptor shows an asymmetric expression pattern as early as HH25–26 in both sexes [Andrews et al., 1997; Nakabayashi et al., 1998]. Epithelial expression of ERα is detected only in the left gonad, although medullary expression is found in both left and right gonads. Crucially, although this pattern of RNA transcription is similar in both males and females, the translated protein displays a sexually dimorphic profile. Immunohistochemistry studies found conspicuous amounts of nuclear ERα protein reflecting the RNA pattern only in the female (fig. 5b), while in the male the protein was barely detectable and cytoplasmic [Andrews et al., 1997; Guioli and Lovell-Badge, 2007]. This female–specific asymmetric ERα pattern was shown to persist at least up to HH39 [Guioli and Lovell-Badge, 2007], and it coincides with the distribution pattern of estrogen target cells defined by radio-immuno assays [Gasc, 1980]. As estrogen is an essential element for ovarian determination in birds (see next chapter), ERα asymmetry may be a primary cause of the different fates of the left and right ovaries.

Because both male and female gonads clearly show distinct L:R morphological and molecular features prior to the appearance of any obvious sexual differentiation, a number of studies focused on the signalling pathway that controls the L:R body axis. Following gastrulation, this pathway operates through a cascade of asymmetric molecular signals initiated at the node and propagated to the lateral plate mesoderm. The downstream target on the left side is the transcription factor PITX2 [Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Gage et al., 1999]. It was found that this gene is expressed exclusively in the epithelium of the left gonad in both sexes from the time the gonadal primordia arise, and that this is maintained at least until HH39 [Guioli and Lovell-Badge, 2007]. When visceral organ heterotaxia was induced using the pharmacological compound Lindane, it was found that gonad situs-specific morphogenesis was also affected [Guioli and Lovell-Badge, 2007]. Moreover, it was shown that the interference of the L:R pathway is mediated via a direct instructive role of PITX2 at organ level, as PITX2 expression in the right gonad epithelium was sufficient to induce the differentiation of a cortex containing meiotic germ cells [Guioli and Lovell-Badge, 2007] (fig. 6).

Two further studies confirmed the role of PITX2 in conferring epithelial left identity [Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

All the L:R molecular differences identified to date are limited to the epithelium and are downstream of PITX2, indicating that PITX2 directly or indirectly affects multiple aspects of gonad epithelial morphogenesis, including the proliferation, adhesion and estrogen signalling properties already described [Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

Specific details of the molecular mechanisms by which PITX2 may control differentiation of the cortex are still poorly understood. It has been shown that components of RA signalling are affected by the gonadal asymmetry established by PITX2, including nuclear receptors activated upon RA binding (RARα and RXRα) and enzymes that are involved in the synthesis and breakdown of RA (RALDH2 and CYP26A1, respectively) [Ishimaru et al., 2008]. By HH27, epithelial RARα and RXRα levels are higher in the right than in the left gonad suggesting that RA signalling is activated preferentially in the right epithelium. This is supported by the fact that RALDH2 and CYP26A1 show a complementary expression pattern with respect to the gonadal epithelia: with RALDH2 expressed on the right and CYP26A1 expressed on the left.

A series of experiments were carried out where beads soaked in RA were implanted in the left side of the embryo. RA did not affect PITX2 expression in the epithelium of the left gonad, but it did down-regulate the expression of ERα, SF1 and CD1 and decreased the rate of proliferation in the epithelium. Conversely, ERα, SF1 and CD1 expression was stimulated in the epithelium of the right gonad by an RA-antagonist [Ishimaru et al., 2008]. The treatments had no obvious effect on medullary tissue. These data suggest that down-regulation of RA signalling within the epithelium allows the epithelium to acquire ‘left identity’, based on the markers analysed. However, this study was performed only up to the widely accepted point of ‘gonadal sex determination’ (HH27–28); therefore, it is not certain that the induced ‘left identity’ would be maintained on the RA–ve/PITX2–ve right side, or if the potential loss of left identity persists on the RA+ve/PITX2+ve left side, as the formation/maintenance of a proper cortex was not assessed. Interestingly, in the period just prior to the expression of the meiotic gene STRA8 and the initiation of meiosis (HH38–41), the somatic cells of the left cortex do express RALDH2 and do not express any CYP26, suggesting that in chick, as in mammals, RA is important for the initiation of meiosis [Smith et al., 2008a; Yu et al., 2013]. At this stage of development, although PITX2 is still expressed, it is down-regulated in comparison to earlier stages [S.G., unpubl.].
As it has been previously shown that PITX2 dosage affects organ morphogenesis [Kozlowski and Walter, 2000; Liu et al., 2001], it is conceivable that the reduced levels of PITX2 found at HH38 may have a different effect on RA signalling, compared to the effect seen at earlier stages. However, the putative late activation of RA signalling observed in the cortex suggests a more complex relationship between RA and L:R identity, and that RA signalling only needs to be temporarily downregulated in the epithelium at early stages of ovary development to allow the formation of the cortex.

It is unclear how PITX2 might control RA signalling. It may be that PITX2 regulates the expression of the genes involved in RA metabolism, as misexpression of PITX2 on the right side caused down-regulation of RALDH2 expression and misexpression of PITX2-engrailed on the left side caused upregulation of RALDH2 expression [Ishimaru et al., 2008].

It has not been established that RA signalling is the only primary downstream target of PITX2 activity: for example, a signalling molecule expressed asymmetrically in the epithelium of the left undifferentiated gonad, and...
which has been shown to be PITX2-dependent, is BMP7 [Hoshino et al., 2005; Guioli and Lovell-Badge, 2007], although the impact of these findings on BMP signalling and on asymmetry is unclear.

In addition, canonical WNT signalling may play a role in ovary development in birds. This is a major determinant of ovarian fate in mammals and is regulated via complex feedback interactions that include Wnt4 and Rspo1 [Zaytouni et al., 2011; Tevosian, 2013]. In the chicken, as in the mouse, these 2 molecules are upregulated in females at the time of gonadal differentiation, suggesting a conserved role in ovarian differentiation. Interestingly, in the chick, these proteins are mostly localised within the developing cortex of the left ovary [Smith et al., 2008b; Ayers et al., 2013]. Although, as yet, there is no direct evidence linking WNT signalling and the functional asymmetry of ovarian differentiation, it is known that WNT signalling and PITX2 are engaged in positive feedback regulation in many systems [Kioussi et al., 2002; Briata et al., 2003; Vadlamudi et al., 2005; Amen et al., 2007; Abu-Elmagd et al., 2010; Zacharias and Gage, 2010; Basu and Roy, 2013].

Finally, PITX2 may directly provide fine-tuning of genes involved in epithelial morphogenesis (RA sensitive or insensitive). Indeed Pitx2 is a known regulator of proliferation through direct activation of specific growth control genes [Kioussi et al., 2002; Baek et al., 2003; Gherzi et al., 2010; Basu and Roy, 2013]. For example, a direct effect on CD1 by PITX2 cannot be excluded as PITX2 has been shown to bind the CD1 promoter in other tissues [Baek et al., 2003]. In addition, a number of studies indicate that PITX2 controls genes involved in the reorganisation of the cytoskeleton, affecting cell spreading, migration and cell-cell-adhesion [Wei and Adelstein, 2002; Campbell et al., 2012].

L:R Asymmetry and Sex (Gonadal) Determination

Our current view of gonadal sex determination is primarily based on work carried out on mammalian models. The so-called bipotential gonad is usually envisaged as a domain of competition between 2 opposing influences, and the initial point of gonadal sex determination is effectively the time when an imbalance is created in favour of one system. It is thought that the cell fate decision made within individual somatic cells is then coordinated to pattern the entire field according to the chosen pathway [Kim and Capel, 2006]. In general, it seems that the molecules that regulate implementation of the male and female differentiation programmes (e.g. SOX9, FOXL2) are conserved across vertebrates, while the primary triggers that initiate these programmes, are not. In mammals, the Y-chromosome gene Sry acts as a trigger for the activation of Sox9, the master regulator of Sertoli cell differentiation. Ovarian differentiation is less well understood, but it seems to involve more than 1 pathway acting in a cooperative manner, including FOXL2 and β-catenin regulatory networks. In the mouse, FOXL2 is only required after birth, whereas in some other mammals (e.g. goat) it is also essential for female sex determination [DeFalco and Capel, 2009; Veitia, 2010; Cutting et al., 2013; Sekido and Lovell-Badge, 2013; Tevosian, 2013].

In recent years it has become evident that, following the initial commitment to the female or male pathway, the gonads retain a plasticity and that even adult gonads may undergo extensive reprogramming upon the loss of key sex regulators. In the mouse, Foxl2/ER are required in the ovary to repress the male factor Sox9 and to maintain female granulosa cell identity, while Dmrt1 is required in the testis to repress Foxl2 and to maintain testis identity, suggesting that the antagonism between Foxl2 and Dmrt1 is critical for the stability of the gonadal sex [Uhlenhaut et al., 2009; Matson et al., 2011].

In birds, it has long been established that the females are the heterogametic sex (ZW sex chromosomes) while the males are homogametic (ZZ); however, the mechanism of sex determination is still poorly understood. The involvement of a sex-specific primary trigger akin to the mammalian Sry gene [Koopman et al., 1991] would require a W-chromosome-encoded ovary-determining gene, but, to date, there is no evidence for the existence of such a factor. An alternative hypothesis proposes that the primary gonadal sex determination trigger is dependent on the level of expression of a Z-chromosome gene(s), with males (ZZ) having higher levels of expression than females (ZW) [reviewed in Clinton, 1998; Cutting et al., 2013]. It is known that the Z-chromosome DMRT1 gene is expressed at higher levels in the male gonads than in the female gonads, from as early as HH25 [Smith et al., 1999]. Moreover, it has also been shown that down-regulation of DMRT1 expression in the male genital ridges in ovo causes a degree of testis-to-ovary transformation, indicating that DMRT1 is required for testis determination [Smith et al., 2009; Chue and Smith, 2011]. These findings support the hypothesis of a Z-chromosome dosage-based mechanism of sex determination in birds and suggest that DMRT1 plays a key role in this process. However, it remains to be demonstrated that a threshold of DMRT1 expression alone is sufficient to trigger testis differentiation.

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and so the involvement of other Z-linked (or even W-linked) factors cannot be excluded. In this context, it has recently been reported that the Z-chromosome gene hemogen is expressed at higher levels in the left and right medulla of male gonads than of female gonads between HH28 and HH35. Overexpression of this transcription factor in female embryos caused decreased FOXL2/P450arom expression and increased SOX9 expression compared to controls and a degree of female to male gonadal sex reversal, suggesting that this gene could also play a role in testis determination [Nakata et al., 2013].

A study generating mixed-sex chimeric chickens recently demonstrated that avian cells have a cell-autonomous sex identity, and this could also support a dosage-based mechanism of gonadal sex determination [Zhao et al., 2010; Clinton et al., 2012]. It was found that individual male (ZZ) cells located in a developing ovary (ZW) were not incorporated into the aromatase-expressing medullary cords. However, if present in sufficient number, these cells differentiated into Sertoli cells and initiated sex-cord formation. Conversely, ZW cells in a developing testis responded by initiating ovarian development and expressing aromatase. Clearly the ‘donor’ cells in these mixed-sex chimeras can correctly interpret the host developmental signals for gonad differentiation, but respond in a donor-specific, cell-autonomous fashion. If the outcome of the developmental process that commits the gonad to a sex-specific pathway was simply a signal that initiates DMRT1 expression from the Z chromosomes in both sexes, then male left and right medullary cells (ZZ) would automatically express twice as much DMRT1 transcript as female cells (ZW). Higher levels of DMRT1 could then lead to the expression of SOX9 and initiate Sertoli cell differentiation.

In chicken, the activation of SOX9 is observed between HH30–31 [Oreal et al., 1998; Moniot et al., 2008] and does coincide with the reorganisation of the primitive cords within the medulla into testis cords containing most of the germ cells. Conversely, the lower level of DMRT1 produced from the single Z chromosome in female cells would result in the activation of FOXL2 in the medulla, possibly leading to repression of DMRT1 and male signals and to the promotion of female signals, including aromatase. Indeed, FOXL2 can activate the aromatase promoter in vitro [Pannetier et al., 2006; Wang et al., 2007; Fleming et al., 2010], and FOXL2 protein expression is female-specific and starts at HH28–29, just prior to the expression of aromatase [Govoroun et al., 2004; Pannetier et al., 2006; Ayers et al., 2013].

The synthesis of gonadal estrogens around the point of sex determination occurs in a large number of vertebrates, including some mammals, and for some of these species, it has been shown that this steroid is a major player in gonadal sex determination/differentiation. In this respect, the chicken is no exception [Shore and Shemesh, 1981; Scheib, 1983; Fadem and Tesoriero, 1986; Crews et al., 1991; Elbrecht and Smith, 1992; Matthiessen and Sumpter, 1998; Coveney et al., 2001; Quirke et al., 2001; Pieu and Dorizzi, 2004; Hudson et al., 2005; Pailhoux et al., 2005; Pettersson et al., 2006; Zha et al., 2008; Barske and Capel, 2010; Pask et al., 2010]. Indeed, using an aromatase inhibitor (fadrozole) to block estrogen synthesis in the female chick embryo leads to an up-regulation of DMRT1 expression from the single Z chromosome, and to increased levels of SOX9 and decreased levels of FOXL2 in left and right medulla [Smith et al., 2003; Hudson et al., 2005]. Although fadrozole treatment almost invariably leads to the differentiation of a testis on the right side of female embryos, the fate of the left gonad is less clear-cut. The left gonad can present as a testis, an ovotestis or an ovary, presumably reflecting the effectiveness of estrogen inhibition. Intriguingly, on the left side it is not uncommon to find embryos with a masculinised medulla juxtaposed to a cortical structure [Vaillant et al., 2001a, b], suggesting that sex-specific differentiation of medulla and epithelium can, to some extent, be uncoupled at the time of gonadal determination. This reinforces the concept that, unlike the situation seen in the mouse, the chick medulla and epithelium are already quite distinct domains at the time of sex determination. Perhaps in some fadrozole-treated embryos, sufficient estrogen is produced to stimulate the initial differentiation of an ovarian cortex, but this is insufficient to prevent the formation of medullary testis cords along the male pathway, a scenario suggesting that estrogen signalling may act as both an antagonist of the male pathway and a promoter of ovarian differentiation.

In most vertebrates, the commitment to one sex results in the development of paired bilateral gonads indicating that, in most cases, the basic L:R asymmetry does not impact on the process of gonadal sex determination and gonadal development. Even in birds, the epithelial L:R asymmetry has little effect on testes differentiation, suggesting that the male pathway can either override or ignore differences between the right and left epithelia. In contrast, morphogenesis of the germinal epithelium is a central event in female gonadal development, and L:R asymmetry is clearly critical for implementation of the ovarian pathway in birds.

Our current understanding of the regulation of female gonadal asymmetry is summarised in figure 7.
Discussion

There are various asymmetrical features of gonadal development in birds; some are present in both sexes while others are restricted to the female. Prior to the point of gonadal sex determination, 2 morphological L:R asymmetries are common to males and females: first, a greater proportion of the circulating PGCs colonise the left gonad than the right, and second, the epithelial layer covering the left gonad is thicker than that covering the right gonad. After gonadal sex determination, a further asymmetry is restricted to females: only the gonad on the left side develops a cortex containing most germ cells committed to meiosis (black dots). Current model of the molecular network involved in the differentiation of the ovarian cortex and affected by the L:R asymmetry pathway via PITX2: In the left epithelium, PITX2 promotes the expression of ERα and SF1 by blocking RA signalling (black lines). This is thought to result in higher proliferation of the epithelium and ability to respond to E2. Other signalling pathways may also be involved including WNT and BMP. By HH38, although PITX2 is still expressed, RA signalling in the cortex is active and is linked to STRA8 activation and initiation of meiosis. Dotted lines indicate possible interactions.
Perhaps in birds, the left ovary performs both roles while the right is limited to acting as an endocrine organ during embryonic development.

Although one obvious possibility is that the development of a reproductive system with only 1 ovary and 1 oviduct is an adaptation to flight, the evolution of this phenomenon is not clear. In most instances, the ability to fly is clearly associated with 1 ovary/1 oviduct – for example, the majority of modern flying birds have only 1 ovary and 1 oviduct, and bird fossils from the Cretaceous period [125 million years ago (mya)] have a single ovary on the left side, while the non-flying dinosaur ancestors of birds had 2 oviducts and (it is assumed) 2 ovaries [Zheng et al., 2013]. However, in other instances this association is not clear-cut. Amongst the group known as the flightless birds (the ratites), that diverged from modern birds between 60–100 mya [Höhn, 1947; Lofts and Murton, 1973; Cooper and Penny, 1997; Clarke et al., 2005; Harshman et al., 2008] and lost the ability to fly, the kiwi has 2 ovaries while all the other ratites have only one [Kinsky, 1971]. Even in some modern birds such as certain types of birds of prey (e.g. Falconiformes), which appeared only around 10 mya, the incidence of paired ovaries is high [Gunn, 1912; Crew, 1931; Shaw, 1938; Kinsky, 1971; Walter, 1979].

This raises the question as to why would a single ovary/oviduct be a useful adaptation to flight. The most popular theory is that this is an issue of weight – rather along the lines of an airline ‘baggage allowance’ – but relating to the ovary with its hierarchy of large maturing follicles as opposed to the weight of the egg [Zheng et al., 2013]. However, most birds have only a short breeding season, and outwith this period the ovaries are small and quiescent, and do not contain mature follicles. Gonadal weight would only be a serious issue during the breeding season and would also apply to males, as it is not only the single ovary in females but also both (internal) testes in males that increase dramatically in size due to the production of gametes [Höhn, 1947; Lofts and Murton, 1973].

An alternative theory to account for the adoption of a single ovary/oviduct system relates to the fragility of the egg during the final stage of development. Between ovulation and lay, the ovum spends around a day in the oviduct acquiring an eggshell. During this period, the egg is clearly susceptible to physical damage, and it has been suggested that the simultaneous presence of 2 or more shelled eggs in close proximity within the abdomen would lead to a reduction in survival rate [Walter, 1979]. Perhaps this susceptibility to damage led to birds adopting a reproductive system with a single oviduct and this in turn led to the retention of only 1 ovary – in a system with 1 oviduct and 2 ovaries, the production of mature follicles would have to be very tightly coordinated. It is noteworthy that even birds with paired ovaries tend to have only 1 oviduct, typically on the left [Kinsky, 1971]. Of course, the evolution of the shelled egg containing all the materials necessary for complete embryonic development requires that very large ova are produced, and so perhaps it is a combination of space restrictions and egg fragility that led to the development of a single ovary/oviduct system in (most) birds, rather than a question of weight. In this context, it is interesting to note that another egg-laying (non-flying) animal, the platypus, also has only a single functional ovary on the left side [Grützner et al., 2008].

The final question concerns the relationship between the formation of 1 ovary on the left side and the basic system of L:R patterning in vertebrates. It is possible that, in the majority of vertebrates, L:R patterning inhibits ovary development on the right side and/or promotes ovary development only on the left side, and that this asymmetry is repressed to generate paired ovaries – along the lines reported to allow bilateral somite development [Vermot and Pourquie, 2005; Duester, 2007]. An alternative possibility is that the basic L:R patterning system imposes only minor difference between the gonads in all vertebrates, and that most birds have an additional mechanism to promote ovary development on the left side and/or inhibit ovary development on the right side. This could again involve RA signalling as discussed.

The question remains unresolved and further functional studies on the role of the epithelial molecular asymmetries are required. The analysis of PITX2 expression and RA signalling in the birds with a high incidence of 2 ovaries would be particularly informative. This should establish the point in the cascade of events in gonad development where asymmetry is overcome in birds with paired ovaries, and potentially shed new light on how the basic L:R asymmetry pathway intersects with ovarian differentiation.

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