ARTICLES

Somatic sex identity is cell autonomous in the chicken

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In the mammalian model of sex determination, embryos are considered to be sexually indifferent until the transient action of a sex-determining gene initiates gonadal differentiation. Although this model is thought to apply to all vertebrates, this has yet to be established. Here we have examined three lateral gynandromorph chickens (a rare, naturally occurring phenomenon in which one side of the animal appears male and the other female) to investigate the sex-determining mechanism in birds. These studies demonstrated that gynandromorph birds are genuine male:female chimaeras, and indicated that male and female avian somatic cells may have an inherent sex identity. To test this hypothesis, we transplanted presumptive mesoderm between embryos of reciprocal sexes to generate embryos containing male:female chimaeric gonads. In contrast to the outcome for mammalian mixed-sex chimaeras, in chicken mixed-sex chimaeras the donor cells were excluded from the functional structures of the host gonad. In an example where female tissue was transplanted into a male host, donor cells contributing to the developing testis retained a female identity and expressed a marker of female function. Our study demonstrates that avian somatic cells possess an inherent sex identity and that, in birds, sexual differentiation is substantively cell autonomous.

Sexual development in vertebrates is thought to be governed by general principles defined in the early to mid-twentieth century[1,2]. These principles state that the sexual phenotype of individuals is dependent on the gonad: male and female somatic cells and tissues are initially sexually indifferent and sexual dimorphism is imposed by the type of gonad that develops. Although these principles have been challenged, most notably by work on songbird neural development[3–6] and marsupial development[7,8], these observations are generally considered as exceptions. In the currently accepted model, gonadal differentiation is triggered in sexually indifferent embryos by the transient action of a sex-determining gene. In mammals, the sex-determining gene is known to be the testis-determining *Sry* gene carried by the male-specific Y chromosome[9]. Although all vertebrates are thought to conform to this general model, with the exception of *Sry* in mammals and *Dmy* in medaka[10,11], no other vertebrate sex-determining genes have been confirmed.

In terms of morphology, birds seem to conform to the mammalian pattern: male and female embryos are sexually indistinguishable until around days 5–6 of incubation (Hamburger and Hamilton[12] (H&H) stage 28/29) when the action of a sex-determining gene is thought to initiate testis or ovary development[13]. However, in birds, not only is the identity of the putative sex-determining gene unknown, the nature of the sex-determining mechanism has not been established. Current theories of sex determination in birds include the presence of an ovary-determining gene on the female-specific W chromosome, and a dosage mechanism based on the number of Z chromosomes (females have one Z and one W sex chromosome whereas males have two Z sex chromosomes)[14]. Currently, the best candidate for a testis-determining gene in birds is *DMRT1* (doublesex and mab-3-related transcription factor 1). Expression of *DMRT1* is restricted to the gonads and it has recently been shown that repressing levels of DMRT1 in male embryos has a 'feminizing' effect on the developing testis[15].

In an attempt to clarify the nature of the sex-determining mechanism in birds, we have investigated the composition of a number of gynandromorph chickens. These birds are rare, naturally occurring phenomena in which one side of the animal appears male and the other female[16]. We investigated these birds with the expectation that this condition resulted from a sex-chromosome aneuploidy on one side of the bird, and that our analysis would provide evidence regarding the nature of the avian sex-determining mechanism. Contrary to expectations, our analysis established that the gynandromorphs were in fact male:female chimaeras, and that the gynandromorphic phenotype was due to ZZ (male) and ZW (female) somatic cells responding in different ways to the same profile of gonadal hormones. These observations led to a series of transcriptome screens and embryonic transplantation studies showing that male and female avian cells possessed an inherent sex identity. Our studies demonstrate that in chickens, gonadal development and the sexual phenotype are largely cell autonomous and not principally dependent on sex hormones.

Gynandromorph birds are mixed-sex chimaeras

We obtained three adult lateral gynandromorph birds (designated G1, G2 and G3) which we maintained and observed over a period of 24 months. These birds occur naturally and it has been suggested that this condition results from the loss of a single sex chromosome at the two-cell stage[17]. All three birds were ISA brown commercial hybrids with sex-linked plumage colour. ISA brown males are heterozygous for the dominant silver and recessive gold genes (*Ss*) and so have white plumage; females possess only the gold gene (*s*-) and have brown plumage. The birds displayed a marked bilateral asymmetry, where one side of the animal appeared phenotypically female and the

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other side phenotypically male (Supplementary Fig. 1). Figure 1 shows a picture of G1 where the right side of the bird is female in coloration (brown) and has a small wattle and small leg spur. In contrast, the left side is male coloured (predominantly white), has a large wattle and a large leg spur, a heavier leg structure and an obviously greater mass of breast muscle, typical of a cockerel. Post mortem, whole tissues from both sides were weighed and measured and samples of all tissues were taken for later analysis. The measurements performed on individual tissues from both sides of all three animals supported the observation that these animals were, at least phenotypically, half male and half female. On the side that appeared male, tissues were larger and heavier and bones were longer and denser than corresponding tissues and bones from the side with a female appearance (Supplementary Table 1). Fluorescent *in situ* hybridization (FISH) analysis using Z and W chromosome probes was performed on preparations of blood cells from all three animals and on multiple preparations of cultured skin cells from both sides of birds G2 and G3. Whereas an autosomal probe demonstrated a diploid chromosome constitution for G1 blood cells, the sex chromosome probes demonstrated that approximately half of gynandromorph G1 cells were female (ZW) and half were male (ZZ) (Fig. 2a). Similar FISH analyses of blood and primary fibroblast cultures from birds G2 and G3 demonstrated that all three animals were composed of a mixture of normal diploid male and female cells (Supplementary Fig. 2 and Supplementary Table 2). Although a recent analysis of a gynandromorph zebra finch[3] demonstrated that both Z-chromosome and W-chromosome containing cells were present, the possibility remained that such animals were composed of a mixture of ZW and Z0 cells. Here we show that gynandromorph birds are genuine male:female chimaeras and provide an explanation for a phenomenon that has been debated for centuries[18].

We next investigated whether the apparent bilateral asymmetry reflected the distribution of ZZ and ZW cells by examining the cellular

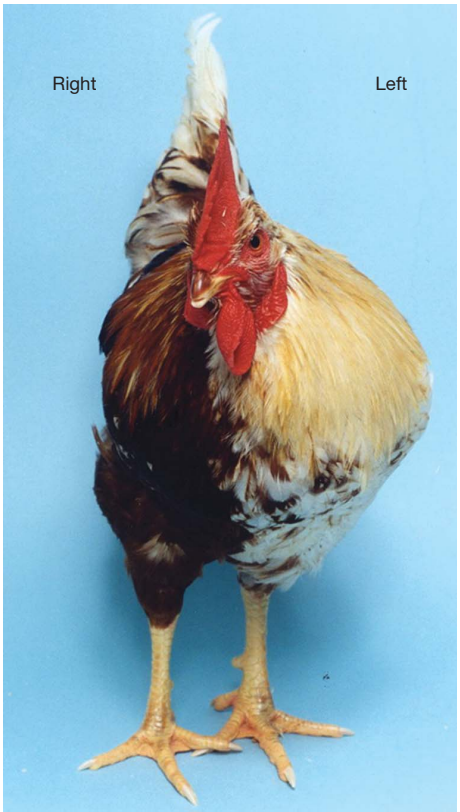

Figure 1 | Image of gynandromorph bird (G1). ISA brown bird where the right side has female characteristics and left side has male characteristics (white colour and larger wattle, breast musculature and spur).

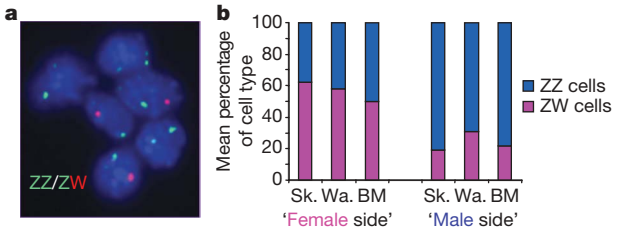

Figure 2 | Male and female cells in gynandromorph birds. a, FISH analysis of sex chromosomes in gynandromorph blood cells. Shown are interphase nuclei prepared from cultured blood cells from gynandromorph G1 hybridized according to standard FISH procedures with probes specific to both the W and Z chromosome (XhoI repeat on W chromosome, and Z chromosome bacterial artificial chromosome (BAC) containing the VLDL receptor gene identified by screening the HGMP chicken BAC library). Erythrocytes were hybridized with probes for Z chromosome (green) and W chromosome (red). Cells contain either two Z chromosomes or one Z and one W chromosome. **b**, Mean relative proportions of ZZ and ZW cells in tissues from male and female sides of gynandromorph birds. The average percentage of ZW and ZZ cells (Supplementary Table 2) in three tissues from the phenotypically female side and from the phenotypically male side of three gynandromorph birds is shown. Tissues from the sides that appear female contain more ZW (female) than ZZ (male) cells, whereas tissues from the sides that appear male are composed predominantly of ZZ cells. BM, breast muscle; Sk., skin; Wa., wattle.

composition of a variety of tissues from both sides of the individual birds. Southern analysis using sex chromosome probes on genomic DNA extracted from multiple tissues revealed that none of the tissues from either side was composed exclusively of either ZZ- or ZW-containing cells; that is, all tissues examined comprised a mixture of both female and male cells (examples shown in Supplementary Fig. 3). Multiple Southern analyses were performed on separate DNA samples extracted from different regions of skin, from wattle and from breast muscle from both sides of all three birds, to quantify the relative proportions of male and female cells. Phosphorimager analyses comparing the hybridization signal obtained from DNA from gynandromorphic tissues with that obtained from known amounts of male and female DNA produced a measure of the relative proportion of male and female cells in each tissue. Figure 2b shows the mean proportion of female and male cells in skin, wattle and breast muscle from the 'male' side and 'female' side of all three birds. It is clear that tissues from the side that appeared female contained more ZW (female) than ZZ (male) cells, whereas tissues from the side that appeared male were composed predominantly of ZZ cells (Supplementary Table 2). Our data establishing the presence of both ZZ- and ZW-containing cells indicate that it is highly unlikely that these birds arise as a consequence of mutation at the two-cell stage of development, and would support the hypothesis that gynandromorphs arise as a result of failure of extrusion of a polar body during meiosis and subsequent fertilization of both a Z- and W-bearing female pronucleus[19].

The development of gonads in the gynandromorph birds was of obvious interest (Supplementary Fig. 4). The type of gonad present did not correspond to the external appearance but rather reflected the cellular composition of the individual organs. The gonads differed for each gynandromorph: G1 contained a testis-like gonad on the left side, G2 contained an ovary-like gonad on the left side, and G3 contained a swollen testis-like structure on the left side (in contrast to G1 and G2, G3 appeared female on the left side and male on the right). The G1 testis-like gonad was composed primarily of sperm-containing seminiferous tubules, whereas the G2 ovary-like gonad was composed predominantly of large and small follicles. The gonad from G3 comprised a mixture of empty tubules and small follicular-like structures (ovo-testis). Southern analyses demonstrated that the morphological appearance of the gonads conformed to the cellular composition in that the structures that appeared to be testis and ovary were composed principally of ZZ- and ZW-containing cells,

respectively, whereas the 'ovo-testis' comprised a mixture of ZZ- and ZW-containing cells.

Although the findings from our gynandromorph analyses are uninformative in terms of elucidating the avian sex-determining mechanism, they do lead to the conclusion that the classical dogma of sex differentiation, where the phenotype is mainly determined by gonadal hormonal secretions, does not apply to birds. These results strongly indicate that the avian phenotype is dependent on the nature of the cells comprising the individual tissue rather than being imposed by the type of gonad formed: both sides of these animals are exposed to an identical profile of gonadal products yet each side responds differently to these stimuli. For example, although it is well established that growth of the wattle is sensitive to testosterone[20], it is clear from Fig. 1 that a major determinant in wattle size is the cellular composition of the tissue, and therefore cellular identity and gonadal hormones both have a significant role in establishing the sexual phenotype of this tissue. Our analyses led us to conclude that male and female chicken somatic cells may have a cell-autonomous sex identity.

Sex differences precede gonadal hormone influences

To investigate whether differences exist between male and female cells independently of any possible gonadal influences we compared the transcriptomes of male and female embryos at developmental stages before the formation of the gonads (data not shown). These analyses identified both messenger RNAs and microRNAs (miRNAs) that were expressed in a sexually dimorphic fashion throughout the embryos at stages before the formation of the gonadal precursor (the genital ridge; H&H stage 21) and well before the generally accepted point of sex determination in the chicken (that is, around day 5/6 of incubation). Screening for mRNAs expressed exclusively in male or in female embryos led to the identification of an mRNA encoded by a W chromosome gene that was expressed ubiquitously in females. This gene was designated *FAF* for female-associated factor and sequences were deposited in the EMBL/GenBank databases (accession numbers AJ606294–AJ606297). Whole-mount *in situ* hybridization analysis of embryos at stages before genital ridge formation showed that *FAF* mRNA is expressed throughout the female embryo as early as 18 h of incubation (H&H stage 4) (Fig. 3a). We also identified a ubiquitously expressed miRNA that is present at levels approximately tenfold higher in males than in females throughout development, including at stages before the expected point of sex determination (Fig. 3b and Supplementary Fig. 5). This miRNA is encoded on the Z chromosome and the sequence has not previously been reported in any other species (*Gallus gallus* mir-2954, accession number AM691163). These observations are in agreement with other studies that have identified sexually dimorphic gene expression in the brain preceding morphological differentiation of the gonads, in both chicken and mouse[21,22]. Although the functions of these particular transcripts are unknown, these findings not only supported the concept that the tissue phenotype was not dependent on gonadal products, but also reinforced the suggestion that the phenotype was defined by inherent differences between the male and female cells.

Chimaeras confirm cell-autonomous sexual differentiation

To test the hypothesis that the male and female cellular composition defines phenotype, we generated embryos containing chimaeric gonads comprised of a mixture of male and female cells. Gonadal chimaeras were generated by transplantation of sections of presumptive mesoderm from green fluorescent protein (GFP)-expressing embryos[23] at developmental stage 12 (day 2) to replace the equivalent tissue of non-GFP embryos at the same stage of development (Fig. 4a). Donor tissue was transplanted only to the left side of recipient embryos as only the left ovary develops fully in the chick. Transplanted embryos were returned to the incubator and allowed to develop until stage 35 (day 9). By stage 35, donor cells were incorporated into tissues on the left side of the embryo in the region between the fore and hind limbs (Supplementary Fig. 6). In addition to the gonads, donor cells were observed in a variety of tissues including skin, muscle, mesonephros, Wolffian duct and Müllerian duct. A minimum of four donor:host chimaeric gonads were generated for each of the four possible combinations: male:male, female:female, male:female and female:male. For each of these donor:host combinations, chimaeras were generated with different levels of donor contribution—ranging from examples where the contribution of donor cells was limited to isolated individual cells dispersed throughout the host gonad, to instances where areas of the host gonad were almost exclusively composed of donor cells. Gonad:mesonephros pairs were collected at stage 35 and longitudinal frozen sections were prepared for confocal microscopy. GFP expression was used to estimate the extent of donor contribution to the individual chimaeric gonads and immunohistochemistry (IHC) analysis was performed with antibodies for both anti-Müllerian hormone (AMH) and aromatase. AMH is a marker of functionally 'male' cells[24] (expressed by precursor Sertoli cells of the sex cords) whereas aromatase is a marker of functionally 'female' cells[25] (expressed by cells in the female medullary region). At stage 35 of development, the male gonad is composed of a thin layer of cortex tissue surrounding a medullary region which contains the developing sex cords (expressing AMH) separated by interstitial connective tissue. In contrast, the female left ovary comprises a greatly thickened cortex surrounding a smaller less-structured medullary region (expressing aromatase). Figure 4b shows the normal expression of AMH and aromatase in stage 35 male and female gonads, respectively. It is clear that the testis is composed almost exclusively of medullary tissue and that AMH is expressed in distinct cord-like structures within this tissue. In contrast, the developing ovary comprises a definitive cortex enclosing a reduced medulla and aromatase is expressed in cells throughout the medulla. Examples of

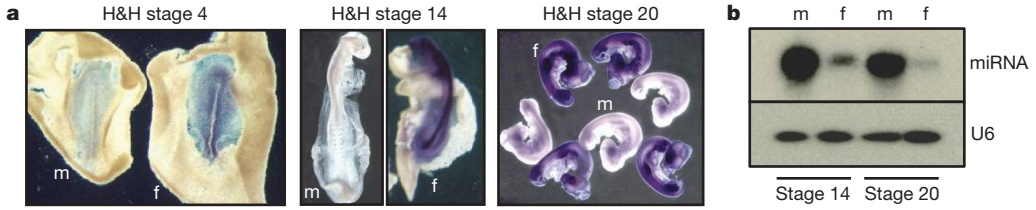

Figure 3 | Sexually dimorphic expression in early chick embryos. **a**, Expression of *FAF* in male and female embryos before development of genital ridge/gonads. Whole-mount ISH showing expression of *FAF* (purple) in embryos at 18 h, 48 h and 72 h of development (H&H stages 4 (original magnification, ×40), 14 (×20) and 20 (×10), respectively). *FAF* is clearly expressed throughout the female embryos at all developmental stages and is not expressed in male embryos. *FAF* is not expressed in extra-embryonic tissues of the female. The *FAF* transcript is encoded by the genomic DNA complementary to the intergenic regions between copies of the W-chromosome repeat gene *Wpkci* (also called *HINTW*)[34,35] and transcribed in the opposite orientation. f, female; m, male. **b**, Expression of novel chicken miRNA (*Gallus gallus* mir-2954). Expression in whole embryos at 48 h (H&H 14) and 72 h (H&H 20) of development is shown. This miRNA is clearly expressed in a sexually dimorphic fashion at stages before the sexual differentiation of the gonads. This miRNA matches sequence present in chicken Z-chromosome BAC clones AC192757 and AC187119. U6 RNA was used as a loading control.

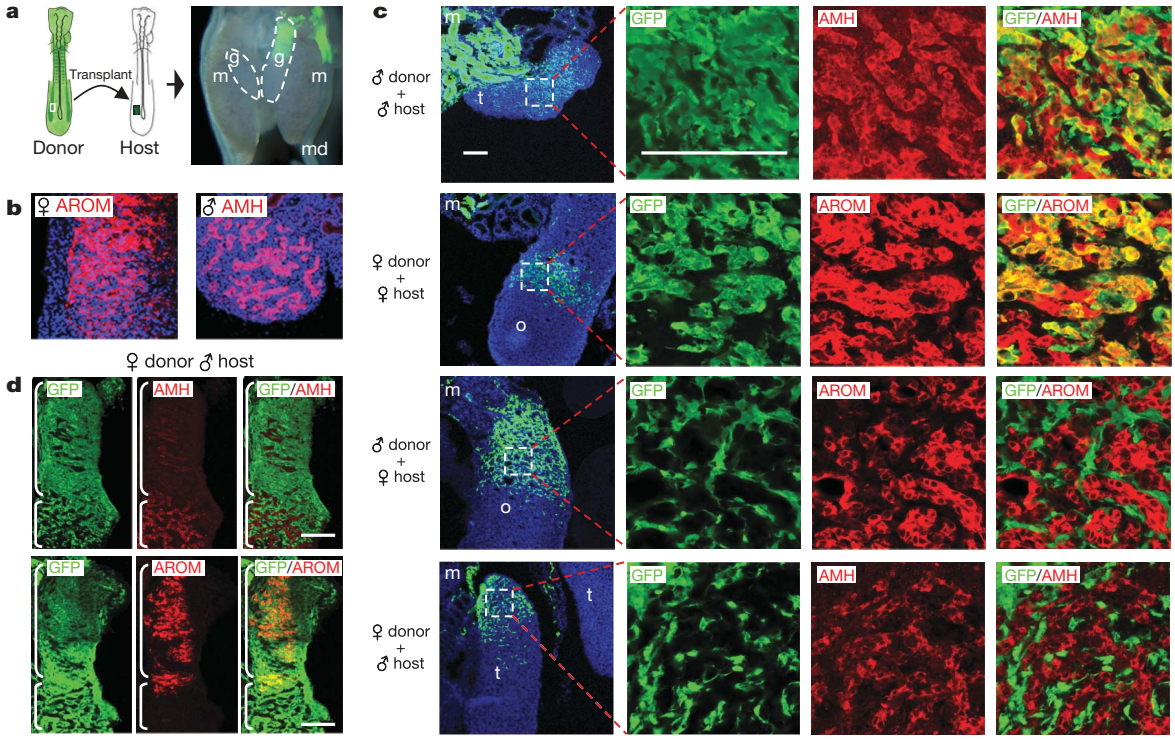

Figure 4 | Expression of male and female markers in chimaeric gonads. **a**, Generation of chimaeras. Left: schematic illustrating transplantation of presumptive mesoderm from GFP-expressing embryo to non-GFP embryo at day 2. Right: image of mesonephros and gonads from chimaeric embryo at day 9 showing donor contribution to left gonad (g), mesonephros (m) and Müllerian duct (md). Original magnification, ×20. **b**, Expression of female and male markers in embryonic gonads. Expression of aromatase (AROM) in ovary and anti-Müllerian hormone (AMH) in testis at day 9 is shown by IHC. Original magnification, ×400. **c**, Integration of GFP-expressing donor cells into host gonads. Panels in the first column show a low-magnification view of sections through host gonads and illustrate the extent of donor cell contribution. Panels to the right show higher-magnification views of highlighted areas. Using IHC, donor cells are marked by GFP (green) whereas expression of AMH and aromatase are shown in red. The fourth column is a merged image of the images from the second and third columns. In same-sex chimaeras, GFP-expressing donor cells co-localize with AMH-expressing and aromatase-expressing cells in host testis and ovary, respectively (yellow/orange in the fourth column). In mixed-sex chimaeras, GFP-expressing donor cells do not co-localize with AMH or aromatase. m, mesonephros; o, ovary; t, testis. **d**, Retention of female donor phenotype in mixed-sex chimaeras. IHC showing expression of AMH (red in top row) and aromatase (red in bottom row) in neighbouring sections from the gonad of a female:male (donor:host) chimaera. Donor contribution is illustrated by GFP (green) expression. The right column shows a merged image of the images in left and middle columns. Regions containing a significant host contribution (defined by the bottom bracket) formed sex-cord-like structures and expressed AMH. Female donor cells were not incorporated into AMH-expressing sex cords, as shown by the lack of GFP and AMH co-localization. Regions composed primarily of female donor cells (defined by top bracket) behaved as ovarian-like tissue and expressed aromatase, as shown by co-localization of GFP and aromatase (yellow/orange). Scale bars in **c** and **d** indicate 100 μm. IHC was performed following standard procedures. Primary antibodies were (1:100) goat anti-human AMH (Santa Cruz Biotechnology), (1:200) mouse anti-human cytochrome P450 aromatase (AbD Serotec) and (1:250) rabbit anti-GFP conjugated to Alexa Fluor 488 (Invitrogen). Secondary antibodies were conjugated to Alexa Fluor 594 (Invitrogen).

all four donor:host combinations of chimaeric gonads are shown in Fig. 4c and Supplementary Fig. 7. It is clear that GFP expression did not affect the ability of donor cells to contribute to host tissues and to function normally: in each case of same-sex chimaeras, either male or female donor cells were integrated into all somatic compartments of the respective host testis and ovary (cortex, sex cords and interstitial tissue). Moreover, when integrated into the appropriate 'functional' compartment, donor male cells expressed AMH and donor female cells expressed aromatase. In contrast, in mixed-sex chimaeras the donor cells did not integrate into the 'functional' structures of the host gonad: female donor cells in host testis medulla were not recruited into the AMH-expressing sex cords and were restricted to the interstitial tissue, whereas male donor cells in host ovary were excluded from the aromatase-expressing structures. In mixed-sex chimaeras the inability of donor cells to form functional host structures was evident regardless of the relative contribution of male and female cells (Supplementary Fig. 7). The fact that female chicken cells in an environment and location that induces testicular development cannot be recruited into the functionally 'male' Sertoli cell compartment, and male cells in an ovary-inducing environment are excluded from a functionally 'female' compartment, strongly supports the suggestion that chicken somatic cells possess a cell-autonomous sexual identity.

This is further supported by a striking example where the degree of the female donor contribution was sufficient to effectively generate an 'ovo-testis' in the host embryo (Fig. 4d). This mixed-sex chimaera contained a gonad with an anterior portion composed almost exclusively of female cells. Whereas the posterior portion contained testis-like medulla with AMH-expressing sex cords, the region composed of female cells did not form sex cords and did not express AMH. Most surprisingly, the female cells in this region expressed aromatase. This demonstrates that although female cells in a male embryo can correctly interpret gonadal location and differentiation signals, they respond in a cell-autonomous manner characteristic of a female genotype (and express aromatase). Our findings are in contrast with those from mammalian mixed-sex chimaeras, where XX cells can become functional Sertoli cells and XY cells can become functional granulosa cells[26,27].

These studies demonstrate that avian somatic cells possess a cell-autonomous sex identity. Our results support and extend previous findings[3] that showed that differences between male and female zebra finch brains were a result of endogenous genetic differences in the brain cells themselves. Our analysis of lateral gynandromorph birds, showing that they are male:female chimaeras, and our experimental generation of embryos with mixed-sex chimaeric gonads, together

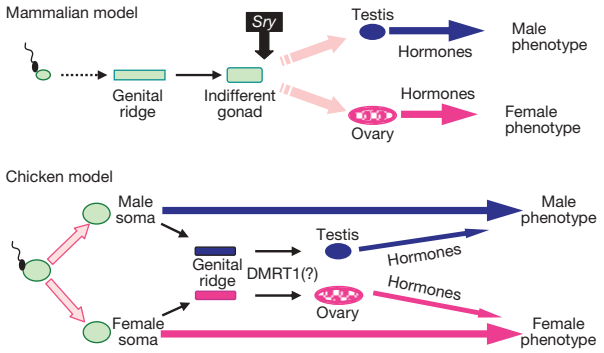

Figure 5 | A novel mechanism of sex determination in the chicken. A sexual identity is genetically imposed on the male and female chicken soma at fertilization and is the major factor in determining the adult sexual phenotype. At the appropriate stage in development, the sexually-dimorphic transcripts underlying the male/female identity trigger expression in the genital ridge of the gene cascade responsible for testis/ovary development. The gonads have limited effects on the sexual phenotype. In contrast, in mammals, gonadal fate is dependent on transient expression of the testis-determining *Sry* gene in the indifferent early gonad. The mammalian gonads have a major influence on the sexual phenotype.

indicate that male and female somatic cells possess a sex identity. These observations indicate that there is a molecular mechanism functioning in every cell that confers a sex-specific identity that influences how individual cells respond to developmental and hormonal signals. We propose that cell-autonomous sex identity is dependent on sexually dimorphic gene expression resulting from the 'dosage compensation' system that operates to equalize the phenotypic effects of characteristics determined by genes on the Z chromosome. Recent evidence has shown that this system in birds is not chromosome-wide and results in a large number of gene expression differences between male and female cells[28–31]. We have estimated that this system of dosage compensation would result in at least 300 non-compensated Z-chromosome genes[31]. Our identification of sexually dimorphic transcripts that are expressed ubiquitously from very early in development adds to these observations. On the basis of our findings, and from evidence of the dosage compensation system in birds, we propose that the overall mechanism of sex determination in birds differs significantly from the mammalian model (Fig. 5). Although sexually dimorphic differentiation of the gonads may be regulated independently from other somatic tissues, we propose that a male or female sex identity is imposed on the chicken soma early in development by sex chromosome transcription and it is this inherent molecular identity that triggers the appropriate testis or ovary gene cascade in the developing genital ridge (for example, via DMRT1 (ref. 15)). Although the gonads clearly have a significant influence on the adult phenotype they do not dictate somatic differences to the same extent as in mammals. It is also possible that elements of such a system are retained in certain mammals: previous studies have shown that, in a marsupial mammal, the wallaby, formation of the mammary gland and scrotum is independent of gonadal hormones[32], and rather than exhibiting transient localized expression, Sry shows widespread expression in multiple tissues well before the point of gonadal differentiation[33]. As Sry-type sex-determining mechanisms have not yet been established for all vertebrate species, it is possible that the model we propose where the phenotype of individual tissues is largely defined by an inherent sex identity of the somatic cells is not restricted to birds.

METHODS SUMMARY

Generation of chimaeric embryos:GFP embryos[23] and ISA brown embryos at H&H stage 11/12 (13–15 somites) were used as donor and host, respectively. The blunt end of donor eggs was pierced to create an air hole and a 'window' cut on the midline. The embryos were removed and pinned on a 3% agarose surface containing 0.5% India ink. Embryos were kept moist by the addition of PBS containing 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin (PBS-Pen/Strep). A strip of presumptive mesoderm flanking presumptive somites 21–23 was removed and stored in CO_2-independent medium (Invitrogen) containing 10% FBS and Pen/Strep. Host eggs were windowed as above and kept moist by the addition of PBS-Pen/Strep. To help visualize somites, sterile India ink (20% in PBS-Pen/Strep) was injected under the host embryos. Using a microneedle, the vitelline membrane and a flap of ectoderm were folded back from the underlying mesoderm. A strip of presumptive mesoderm was then removed from the host embryo taking care to leave the endoderm intact. The GFP-donor tissue was then inserted into the host site and the ectodermal flap replaced. Two millilitres of albumen was then withdrawn from the host eggs using a hypodermic syringe. Transplanted eggs were tightly sealed with tape and incubated at 37 °C in a humidified incubator. All other methods are standard.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.Z. and D.M. performed transplantation studies, transcriptome screens, Southern analyses and general molecular biology. S.N. performed immunostaining, H.A.M. performed FISH analyses and P.M.H. performed dissections and post-mortem measurements. M.J.M. performed ISH and suggested transplantation strategy and P.D.L. obtained gynandromorph birds. Overall project was conceived by M.C. and H.M.S. M.C. carried out day-to-day supervision and wrote the manuscript. All authors edited the manuscript.

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METHODS

Embryo transplantation. Generation of chimaeric embryos:GFP embryos[23] and ISA brown embryos at H&H stage 11/12 (13–15 somites) were used as donor and host, respectively. The blunt end of donor eggs was pierced to create an air hole and a 'window' cut on the midline. The embryos were removed and pinned on a 3% agarose surface containing 0.5% India ink. Embryos were kept moist by the addition of phosphate buffered saline (PBS) containing 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin (PBS-Pen/Strep). A strip of lateral plate mesoderm flanking presumptive somites 21–23 was removed and stored in CO_2-independent medium (Invitrogen) containing 10% FBS and Pen/Strep. Host eggs were windowed as above and kept moist by the addition of PBS-Pen/Strep. To help visualize somites, sterile India ink (20% in PBS-Pen/Strep) was injected under the host embryos. Using a microneedle, the vitelline membrane and a flap of ectoderm were folded back from the underlying mesoderm. A strip of lateral plate mesoderm was then removed from the host embryo, taking care to leave the endoderm intact. The GFP-donor tissue was then inserted into the host site and the ectodermal flap replaced. Two millilitres of albumen was then withdrawn from the host eggs using a hypodermic syringe. Transplanted eggs were tightly sealed with tape and incubated at 37 °C in a humidified incubator.

Immunostaining. Immunohistochemistry was carried out as described previously[36]. Briefly, tissues were fixed in 4% paraformaldehyde for 2 h at 4 °C, equilibrated in 15% sucrose then embedded in 15% sucrose plus 7.5% gelatin in PBS, pH 7.2. Sections, 15 μm thick, mounted on Superfrost slides (Menzel) were washed for 30 min in PBS at 37 °C and blocked in PBS containing 10% donkey serum, 1% BSA, 0.3% Triton X-100 and 0.05% Tween 20 for 2 h at 22–24 °C. Incubation with primary antibodies was carried out overnight at 4 °C, followed by washing in PBS containing 0.3% Triton X-100 and 0.05% Tween 20, and then incubation with secondary antibodies for 2 h at room temperature. After washing, the sections were treated with Hoechst solution ($10\,\mu g\,ml^{-1}$) for 5 min to stain nuclei.

Fluorescent *in situ* hybridization (FISH). FISH analysis of metaphase or interphase preparations of chicken cells was performed by standard procedures[37]. BAC clones containing the VLDL receptor, aldolase B, *CHRN* or *SCII* genes were identified by screening the HGMP chicken BAC library and used to identify Z chromosomes. A probe for the W chromosome was prepared by polymerase chain reaction (PCR) amplification of a portion of the XhoI repeat region from the W chromosome[38]. After gel purification, the probe was labelled by incorporation of either biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche) during a further round of PCR. BAC DNA was prepared using Qiagen plasmid columns following recommendations for low-copy plasmid purification. Biotin-16-dUTP and digoxigenin-11-dUTP were incorporated into BAC DNA by nick translation and labelled probes were concentrated by precipitation in the presence of 5 μg of salmon sperm DNA as a carrier and 2 μg of sonicated chicken genomic DNA as competitor. The pellet was resuspended in 15 μl of hybridization mix, denatured and pre-annealed for 15 min at 37 °C to block repetitive sequences.

Whole-mount *in situ* hybridization. Chicken embryos and isolated gonads were fixed in 4% paraformaldehyde for 1 h and whole-mount *in situ* hybridization was carried out as described previously[39]. Digoxigenin-labelled probes were prepared from linearized plasmid clones using a Roche DIG RNA labelling kit to incorporate digoxigenin-11-UTP by *in vitro* transcription with SP6 and T7 RNA polymerases.

RNA preparation. Total RNA was extracted from pools of male and female chick embryos and tissues using RNA-Bee (AMS Biotechnology) according to the manufacturer's instructions.

Differential display. RNA expression profiles in male and female embryos were compared by differential display reverse transcription PCR (DDRT–PCR). Embryos were sexed[38] and pools of RNA from male and female embryos generated. DDRT–PCR was performed as described previously[40].

miRNA library construction. Low-molecular-mass RNAs (<40 nucleotides long) were isolated from total RNA by the use of a flashPAGE fractionator (Ambion). MicroRNA libraries were constructed essentially as described previously[41,42].

MicroRNA northern analysis. Five micrograms of total RNA was separated by electrophoresis through a 15% TBE/urea polyacrylamide gel (Bio-Rad) before transfer to Hybond-N^+ membrane (GE Healthcare). Locked nucleic acid (LNA) oligonucleotides antisense to the mature miRNA were end-labelled (mirVana Probe and Marker kit, Ambion) with ^{32}P-dATP (Perkin-Elmer) and hybridized to membranes containing miRNAs. Hybridization was carried out overnight in ULTRAhyb-oligo (Invitrogen) at 42 °C and membranes washed at 63 °C in 0.1× SSC/0.1% SDS[43] (22 °C below the estimated melting temperature of the LNA, 85 °C).

Southern analysis. High-molecular-mass genomic DNA was extracted from tissues of embryonic and adult male and female chickens by standard phenol-chloroform procedures[43]. DNA was digested with restriction endonucleases, subjected to electrophoresis on a 1% TBE gel and transferred to Hybond-N membrane. Probes labelled with ^{32}P-dCTP were hybridized by standard procedures and signal was recorded on high-sensitivity film (Kodak) and by phosphorimager analysis.

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