Vertebrate sex determination: many means to an end

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The differentiation of a testis or ovary from a bipotential gonadal primordium is a developmental process common to mammals, birds and reptiles. Since the discovery of SRY, the Y-linked testis-determining gene in mammals, extensive efforts have failed to find its orthologue in other vertebrates, indicating evolutionary plasticity in the switch that triggers sex determination. Several other genes are known to be important for sex determination in mammals, such as SOX9, AMH, WT1, SF1, DAX1 and DMRT1. Analyses of these genes in humans with gonadal dysgenesis, mouse models and using in vitro cell culture assays have revealed that sex determination results from a complex interplay between the genes in this network. All of these genes are conserved in other vertebrates, such as chickens and alligators, and show gonad-specific expression in these species during the period of sex determination. Intriguingly, the sequence, sex specificity and timing of expression of some of these genes during sex determination differ among species. This finding indicates that the interplay between genes in the regulatory network leading to gonad development differs between vertebrates. However, despite this, the development of a testis or ovary from a bipotential gonad is remarkably similar across vertebrates.

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Gonadal development in vertebrates

Mouse

The first sign of gonadogenesis in mice is the formation of the urogenital system from the intermediate mesoderm, which begins at about day 9.0–9.5 after mating. At about day 10.5 after mating, the paired urogenital system runs much of the length of the embryo and fills most of the coelomic cavity. The urogenital system is composed of three segments from anterior to posterior: the pronephros (includes the adrenal primordium), the mesonephros (the embryonic kidney) and the metanephros (the primordium of the definitive kidney). The Wolffian duct is derived from lateral mesoderm and runs the length of the urogenital system. The Müllerian duct appears between day 11.5 and day 12.5 after mating from invagination of surface epithelium from the mesonephros and runs parallel to the Wolffian duct. In females, the Müllerian duct differentiates into the oviduct, uterus and upper parts of the vagina, but is regressed in males by the action of the anti-Müllerian hormone, AMH. In females, the Wolffian duct will mostly degenerate, but in males, testosterone secreted by testicular...
Leydig cells will induce the Wolffian duct to differentiate into the epididymis, vas deferens and seminal vesicles.

From about day 10.5 after mating, the bipotential gonadal primordium arises from the ventromedial surface of the mesonephros as a proliferating layer of coelomic epithelial cells. In male mice, expression of Sry begins at day 10.5 after mating, reaches a peak at day 11.5 after mating and then ceases abruptly by day 12.5 after mating (Hacker et al., 1995). The exact mode of action and immediate targets of SRY are unknown, but the consequence of Sry expression is the rapid induction of morphological changes in male gonads, which clearly distinguishes them from female gonads by day 12.5 after mating (Fig. 1). At this stage, the male gonad is twice the size of the female gonad and is composed of highly structured testis cords surrounded by interstitium. In males, the germ cells are enclosed by the supporting cell lineage, the Sertoli cells (the supporting cells in the ovary are the granulosa/follicle cells). The Sertoli cells are surrounded by basal lamina and peritubular myoid cells, which result in the characteristic cord morphology of the testis. The interstitium of the testis includes the steroidogenic cell lineage, the Leydig cells (theca cells in the ovary) and prominent vascularization. In contrast, the female gonad at day 12.5 after mating is smaller and less well organized than the testis (Fig. 1). In the developing ovary at day 12.5 after mating, the germ cells reside in the interstitium with the supporting and steroidogenic cells, and no prominent vascularization or structural tissue is visible. The appearance of ovigerous cords (egg clusters) in the ovary occurs at about day 15.5 after mating and the oocyte becomes surrounded by granulosa cells to form the primordial follicle just before birth.

One of the most important functions of Sry is thought to be the induction of differentiation of Sertoli cells in the testis. Sry induces increased proliferation of coelomic epithelial cells, which can be first detected from day 11.5 after mating (Schmahl et al., 2000). Sertoli cells and some of the interstitial cells derive from the proliferating coelomic epithelial cells that delaminate from the surface and migrate into the gonad (Karl and Capel, 1998). Sry also induces the migration of cells from the mesonephros into the developing gonad (Capel et al., 1999). These mesonephrically derived cells become the peritubular myoid cells, which interact with the Sertoli cells to lay down the basement membrane, thereby forming the testis cords. Mesonephrically derived cells also make up the vasculature and other regions within the interstitium (Martineau et al., 1997).

Germ cells behave very differently during male and female gonadogenesis. In both sexes, the germ cells migrate from the allantois to the coelomic epithelium of the gonad via the gut mesentery between day 9.5 and day 11.0 after mating, where they proliferate until day 12.5 after mating. At this stage in the male, the germ cells are enclosed within the testis cords and enter mitotic arrest. The germ cells in females continue to undergo mitosis until day 13.5 after mating, when they enter meiotic arrest (McLaren and South, 1997). Meiotic germ cells in females are essential for formation and maintenance of the ovarian follicles (McLaren, 1995). The interaction between germ cells and the supporting cells is thought to play a critical part in sex determination. Sertoli cells that surround the germ cells in the testis cords are thought to prevent the male germ cells from entering meiosis.

**Chicken**

At day 3.5 (stage 22) in chicken embryogenesis, the gonadal primordium begins forming. By day 5.5 (stage 28), the bipotential gonad has formed and both left and right

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Fig. 1. Schematic diagram of a cross-section through an embryonic mouse testis and ovary with adjacent mesonephros at day 12.5 after mating. MD: Müllerian duct; WD: Wolffian duct. In the male gonad, the testis cords are composed of the peritubular myoid cells, the Sertoli cells and the germ cells.

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urogenital systems appear identical in males and females. The sexes can be distinguished morphologically by day 6.5 (stage 30) and testis cord formation is complete in males by day 7.5 (stage 32). One of the striking differences between mammalian and avian gonadogenesis is the left–right asymmetry of chicken gonads. By day 8.5 (stage 35), only the left ovary of the female has developed (the right regresses to a vestige) and the right testis of the male is slightly smaller than the left. In both sexes, there are more germ cells in the larger left gonad. The bipotential gonad in chickens is composed of proliferating coelomic epithelial cells that contain most of the germ cells and an underlying medulla. During male gonadogenesis, there is a proliferation of cells within the medulla and a reduction in the thickness of the coelomic epithelium. Sertoli cells differentiate within the medulla and enclose the germ cells in characteristic testis cords. In females, the coelomic epithelial cells proliferate to form the cortex, which contains the germ cells, whereas the medulla fragments (for a review, see Smith and Sinclair, 2001).

Alligator

The sex of many reptiles, including that of all crocodilians so far examined, is determined by temperature. For the American alligator, *Alligator mississippiensis*, embryogenesis in eggs incubated at 33°C produces all male offspring and takes approximately 64 days (Lang and Andrews, 1994). In contrast, embryogenesis in eggs incubated at 30°C produces all female offspring and takes approximately 70 days. At 33°C, the first signs of testis differentiation are seen between day 28 and day 34 (stages 21–23), with the appearance of pre-Sertoli cells within the medulla. These cells proliferate and differentiate to initiate testis cord formation and enclose the germ cells between day 35 and day 41 (stages 23–24) (Smith and Joss, 1993). Ovarian differentiation at 30°C occurs later, from day 39 (stage 22), with proliferation of the germ cells and pre-granulosa cells in the cortex. Fragmentation of the medulla and formation of oogenital nests within the cortex occur by day 43 (stage 23). The temperature-sensitive period for sex determination in alligators occurs over a period of about 10 days, between stage 21 and stage 24. After this time, the morphogenesis of the gonad will be irreversibly committed to either the male or female fate.

**Genes involved in gonadal differentiation in vertebrates**

Many genes affecting sex determination have been described in mice (for a review, see Swain and Lovell-Badge, 1999). Many of these genes were cloned in an attempt to identify the genes responsible for gonadal dysgenesis in humans, thus defining their role in the process of sex determination. The function of most of these genes has been further studied using mouse transgenic models and cell culture assays, revealing that sex determination results from the interplay of genes in a complex network (Fig. 2).

![Gene regulatory pathways during gonadogenesis](image)

*Fig. 2. Known and postulated gene regulatory pathways during gonadogenesis. Upregulated genes are depicted in bold. Arrow on line indicates positive regulation. Bar on line indicates inhibitory interaction. Solid lines indicate strong interactions and dashed lines indicate weak interactions. SFI: steroidogenic factor 1; WT1: Wilms' tumour suppressor gene; SRY: sex-determining region on the Y chromosome; DMRT1: doublesex- and mab-3-related transcription factor 1; SOX9: Sry-like HMG box; DAX1: dosage sensitive sex-reversal-adrenal hypoplasia congenita-critical region of the X chromosome, gene 1; AMH: anti-Müllerian hormone.*
SOX9, AMH, WT1, SF1 and DMRT1, which are broadly male- or testis-specific and are expressed during or before testis determination in mice, and DAX1, which is upregulated in the female gonad during the period of sex determination in mice, will be discussed.

SOX9 and AMH

The commitment to Sertoli cell differentiation in the mouse testis is considered the most significant aspect of Sry function. The transcription factor, Sox9 (Sry-like HMG box), is the earliest known marker of differentiating Sertoli cells and is therefore postulated to be an immediate downstream target of Sry, although this has not yet been shown experimentally. Anti-Müllerian hormone (AMH) is the first known protein to be secreted by differentiated Sertoli cells in the testis. Thus, Sox9 and Amh are two key components of mammalian testis determination and testis differentiation, respectively.

Humans with heterozygous mutations in SOX9 develop a severe bone disorder, called campomelic dysplasia. Seventy-five per cent of XY campomelic dysplasia patients display some degree of gonadal dysgenesis, indicating that SOX9 may have an important role in testis development. In contrast, ovarian development in XX campomelic dysplasia patients is normal (Foster et al., 1994). Moreover, the consequences of heterozygous SOX9 mutations are thought to result from haploinsufficiency rather than dominant negative effects, supporting the idea that dosage of SOX9 is critical for testis determination in humans (Olney et al., 1999).

SOX9 is a member of the large family of developmentally regulated SOX genes that are related by virtue of sharing > 50% amino acid identity with the DNA-binding HMG box of SRY (Prior and Walter, 1996). Apart from the DNA-binding HMG box, SOX9 in mice and humans contains two transcriptional activation domains, indicating that it is a typical transcription factor (Wright et al., 1995).

In mice, Sox9 expression is first detected in the bipotential gonads of both sexes at day 10.5 after mating (Fig. 3a; Kent et al., 1996). However, soon after the onset of Sry expression, Sox9 expression is upregulated in the male gonad and downregulated in the female gonad, so that from day 11.5 after mating, no Sox9-specific mRNA can be detected in the female gonad (Kent et al., 1996; Morais da Silva et al., 1996). At day 11.5 after mating in XY gonads, SOX9 protein is detected in the cytoplasm and nucleus of Sertoli cell precursors, whereas from day 11.5 after mating SOX9 is detected only in the nucleus of Sertoli cells (Morais da Silva et al., 1996). After birth, Sox9 expression persists in the Sertoli cells through to adulthood, but it is never detected in the ovary.

Mutations in SOX9 result in sex reversal and demonstrate that SOX9 is necessary for testis determination. However, SOX9 is also sufficient for testis determination, as an extra dose of SOX9 resulting from a chromosomal duplication causes male sexual development of an XX individual (Huang et al., 1999). This has been further demonstrated in transgenic XX mice overexpressing Sox9, which develop normal testes (Vidal et al., 2001). To date, there are no reports of the gonadal phenotype of transgenic mice carrying null mutations in SOX9.

In mice, Amh expression is first detected from day 11.5 after mating in the male gonad, as the differentiated Sertoli cells start to align into cords (Fig. 3a; Hacker et al., 1995). From day 12.5 after mating, Amh is highly expressed in males and cannot be detected in females (Münsterberg and Lovell-Badge, 1991). Amh expression is downregulated in males after birth and is not detected in females until day 6 after birth. Mice carrying homozygous deletions in the Amh coding region have normally developed testis, but are infertile due to persistence of Müllerian-derived structures that interfere with sperm transfer (Behringer et al., 1994). Therefore, although Amh is important for the formation of secondary sexual characteristics, it is not required for testis determination.

Several lines of evidence indicate that AMH transcription may be positively regulated by SOX9 (Fig. 2). First, the AMH promoter contains a consensus HMG-binding site that binds SOX9 in vitro (de Santa Barbara et al., 1998). AMH is positively transcriptionally regulated by SOX9 in transiently transfected cultured cells (de Santa Barbara et al., 1998). Transgenic mice carrying mutations in both copies of the putative SOX9-binding site in the endogenous Amh gene have a phenotype indistinguishable from that of mice carrying deletions of the Amh coding region (Arango et al., 1999). Finally, some XY sex-reversed humans with mutations in SOX9 have Müllerian duct-derived structures and are therefore unlikely to have produced AMH (Foster et al., 1994). It should be noted that although the evidence that SOX9 regulates AMH is compelling, it is not conclusive, as SOX9 has not been shown to bind the AMH promoter in vivo. The AMH promoter also has binding sites for the SF1 transcription factor and GATA family members (Shen et al., 1994; Viger et al., 1998). SF1 interacts with SOX9 to transactivate the AMH promoter in vitro (de Santa Barbara et al., 1998). However, although targeted disruption in mice of the SF1 binding site in the endogenous Amh promoter reduces amounts of Amh mRNA by more than threefold compared with wild-type controls, Müllerian duct regression proceeds as normal (Arango et al., 1999). This finding demonstrates that very low amounts of AMH permit its function and highlights the importance of complete repression of Amh expression during female gonadal development.

SOX9 is highly conserved at the amino acid level and the gene shows testis-specific upregulation in all vertebrates examined to date. This finding indicates that SOX9 may represent an ancestral sex-determining gene and that mammals have subsequently evolved SRY as a Y-linked switching mechanism. Several studies have examined the expression of SOX9 in chickens using RT–PCR and RNA in situ hybridization on whole mounts and sections with some conflicting results regarding its onset of expression (Fig. 3b;
Fig. 3. Relative gene expression in male and female gonads during the period of sex determination of embryogenesis in (a) mouse, (b) chicken and (c) alligator. Dotted lines indicate low expression; thin lines indicate moderate expression and thick lines indicate strong expression. The relative levels of expression for a given gene are comparable between the sexes of a given species and not between species or between genes in the same species. SRY: sex-determining region on the Y chromosome; SOX9: Sry-like HMG box; AMH: anti-Müllerian hormone; WT1: Wilms' tumour suppressor gene; SF1: steroidogenic factor 1; DAX1: dosage sensitive sex-reversal-adrenal hypoplasia congenita-critical region of the X chromosome, gene 1; DMRT1: doublesex- and mab-3-related transcription factor 1; dpc: days post coitus; TC: testis cord.
Kent et al., 1996; Morais da Silva et al., 1996; Oreal et al., 1998; Smith et al., 1999a). Onset of SOX9 expression in both sexes from stage 25 was observed using RNA in situ hybridization on whole mounts (Morais da Silva et al., 1996). However, using RNA in situ hybridization on sections, SOX9 was first detected in males from stage 28 onwards (Oreal et al., 1998). However, two other studies (one using RT–PCR) failed to detect SOX9 expression in male gonads before stage 30 or in the female gonad at any stage (Kent et al., 1996; Smith et al., 1999a). The consensus from these studies is that SOX9 mRNA is not detected in the female gonad, although it is strongly expressed in the male gonad from stage 30. The inconsistencies in the interpretation of the onset of SOX9 expression may reflect differences in the methods and probes used by the different groups.

In alligators, RT–PCR and RNA in situ hybridization on sections demonstrate that expression of SOX9 mRNA is upregulated in the gonads of embryos raised at male-producing temperatures during stage 23 (Fig. 3c; Western et al., 1999a). The male-specific upregulation of SOX9 expression occurs at the end of the temperature-sensitive period, after Sertoli-like cells have started aligning into cords. Therefore, SOX9 upregulation in the male alligator occurs about 10 days after the signal to initiate male-specific differentiation of the gonad (Western et al., 1999a).

In chickens, AMH expression can be detected from stage 25 in both sexes, at a time when SOX9 expression cannot be detected (Fig. 3b; Oreal et al., 1998). AMH expression is strongly upregulated in males from stage 28, at a time when weak SOX9 expression is first being detected. However, at stage 28 in females, weak AMH expression can still be detected within the inner part of the gonad (the medulla), whereas SOX9 expression cannot be detected (Oreal et al., 1998). In another study using RT–PCR, AMH expression was detected from stage 28 in both male and female gonads, before first detection of SOX9, and was strongly upregulated in males at stage 30, when SOX9 expression was first detected (Smith et al., 1999a). At all times, AMH expression was much higher in males than in females. Both studies in chickens indicate that the timing of AMH expression precedes that of SOX9 in the developing gonad by about 1 day and that weak AMH expression in females occurs in the absence of SOX9 expression.

In alligators, AMH expression also appears to precede SOX9 expression (Fig. 3c; Western et al., 1999b). RT–PCR and RNA in situ hybridization on serial sections of alligator gonads were used to show that AMH expression begins in males at stage 22 and reaches a peak at stage 24, whereas SOX9 expression was not detected until stage 23.5, 8 days after the onset of AMH expression. As with mice and in contrast to chickens, AMH expression was not detected in female gonads at any stage during alligator gonadogenesis.

In summary, SOX9 appears to have a pivotal role in the initial steps of Sertoli cell differentiation in mice, yet it is unlikely to perform the same function in other vertebrates, such as chickens and alligators. In fact, in alligators, pre-Sertoli cells appear from stage 21, approximately 2 weeks before SOX9 can be detected. In mice, Sox9 expression precedes Amh expression by about 24 h and Sox9 is likely to regulate Amh directly. In chickens and alligators, AMH expression precedes SOX9 expression by about 24 h and 10 days, respectively, making it highly unlikely that SOX9 initiates AMH expression. However, in both chickens and alligators strong upregulation of AMH in males is coincident with the onset of SOX9 expression. This finding indicates that although SOX9 does not initiate AMH in these species, it may have a role in its subsequent upregulation. Moreover, in both mice and chickens, AMH expression in females occurs in the absence of SOX9 expression.

In mice and alligators, morphological changes that distinguish the male gonad from the female gonad precede AMH expression, indicating that AMH expression is a good marker for differentiated Sertoli cells. In chickens, AMH is first detected at stage 25 (or stage 28), 48 (or 24) h before any histological sign of testis morphogenesis (at stage 30). If AMH expression is a universal marker for differentiated Sertoli cells, then in chickens Sertoli cells must differentiate before stage 25, which would place the sex-determining event in chickens before stage 25 (day 4.5). Alternatively, Sertoli cell differentiation may occur closer to the time of morphological differentiation, at about the time of onset of SOX9 expression between stage 28 and stage 30. In the latter scheme, AMH could be expressed in both Sertoli and granulosa cell precursors and the low amounts would not be incompatible with female development. Indeed, AMH in female chicken gonads is postulated to be required for regression of the right Müllerian duct.

Wilms’ tumour suppressor gene (WT1)

WT1 is required in mammals to establish the bipotential gonad and also plays a later role in testis development. WT1 is mutated in patients with Denys–Drash and Frasier syndromes. Individuals with these syndromes display abnormal urogenital development and XY sex reversal, supporting a role for WT1 in gonadogenesis and testis determination (Pelletier et al., 1991a; Barbaux et al., 1997).

In mammals, WT1 is a complex gene comprising ten exons that can generate 24 possible isoforms by use of alternative start sites, splicing and RNA editing. Alternative splicing in exon nine results in isoforms with or without three amino acids, KTS, between the third and fourth zinc fingers. The KTS triplet results in the loss of DNA binding by the fourth zinc finger. The +KTS isoforms are commonly localized with splicing factors and may play a role in RNA processing, whereas the –KTS isoforms are considered transcription factors that can activate or repress transcription. Humans with Frasier syndrome have a splice-site mutation in WT1 that results in the production of only –KTS isoforms.

In mice, WT1 is first detected from day 9.5 after mating throughout the intermediate mesoderm, encompassing the primordial mesonephros, gonads, kidneys and adrenal gland (Fig. 3a; Armstrong et al., 1992). In the bipotential
gonad at day 10.5 after mating. WT1 expression is detected in the somatic cells of the coelomic epithelium of both sexes, then later in the Sertoli cells of the male gonad and in the granulosa and epithelial cells of the female gonad (Pelletier et al., 1991b). During gonadalgenesis in the rat embryo, RNase protection was used to show that concentrations of both WT1 isoforms are similar between males and females (Nachtigal et al., 1998).

The WT1–KTS isoforms can bind and transactivate the SRY and Dax1 promoters in vitro (Fig. 2; Kim et al., 1999; Hossain and Sanders, 2001). Although the WT1–KTS isoforms cannot bind to the AMH promoter, it has been shown to synergize with SF1 to activate AMH transcription in vitro (Nachtigal et al., 1998). In XX individuals, DAX1 is thought to interfere with the interaction of SF1 and WT1, thus repressing male-specific development (Fig. 2; Nachtigal et al., 1998).

Knockout mice carrying homozygous deletions of Wt1 die during mid-gestation and lack gonads, kidneys and adrenal glands, and have heart and spleen defects. Transgenic mice carrying only +KTS (–KTS isoforms ablated) or only –KTS (+KTS isoforms ablated) isoforms of WT1 die shortly after birth from kidney defects (Hammes et al., 2001); however, the gonads of these mice display markedly different phenotypes, which is indicative of distinct functions for each isoform in gonadal development and sex determination. The gonads of mice carrying only +KTS isoforms fail to develop beyond streaks and undergo apoptosis. Patches of cells in the streak gonads of XY individuals still express some markers of testis determination (such as Sox9 and Amh), indicating that –KTS isoforms may be required for formation of the bipotential gonad rather than testis determination. Mice expressing only –KTS isoforms develop gonads, but all XY individuals are sex-reversed, indicating that +KTS isoforms may be essential for testis determination, but not for ovarian development or formation of the bipotential gonad. The expression of Sry is markedly lower in mice carrying only –KTS isoforms, indicating that +KTS isoforms may be normally required for Sry expression (Hammes et al., 2001). This observation is consistent with the phenotype of XY Frasier patients, who develop sex-reversed gonads, but is in contrast to data obtained from in vitro studies indicating that only –KTS isoforms can transactivate the SRY promoter (Hossain and Saunders, 2001). It would appear that –KTS isoforms are essential for formation of the bipotential gonad, but that +KTS isoforms are required to allow enough expression of Sry to ‘tip the balance’ towards testis differentiation.

In chickens, RT–PCR was used to show that there is high expression of WT1 in both male and female gonads from stage 28 to stage 40, before and during gonadal differentiation (Fig. 3b; Smith et al., 1999a). In alligator gonads, WT1 expression can be detected by RT–PCR at stage 20, and expression is high from stage 23 at both male- and female-producing temperatures, but like in chickens, there is no sexual dimorphism in expression (Fig. 3c; Western et al., 2000). These studies did not distinguish between the different isoforms of WT1 that may be critical for sex determination.

In summary, the expression of WT1 in the early stages of chicken and alligator gonadalgenesis supports a conserved function for WT1 across vertebrates during the time at which establishment of the gonadal primordium occurs, before sex determination. In mice, the different isoforms play distinct roles in gonadalgenesis and testis determination, and there is now good evidence from studies in vivo that WT1 enhances expression of Sry. At present, there are no data from other vertebrates addressing a role for WT1 inactivating the expression of the sex-determination switch or in the sex-specific regulation of downstream genes.

Steroidogenic factor 1 (SF1)

SF1 (Ad4BP) is required in mammals for formation of the primary steroidogenic organs: the adrenal gland and the gonad. SF1 is also likely to have a role in testis development. Mutation in human SF1 results in XY gonadal dysgenesis and adrenal failure (Achermann et al., 1999). SF1 is an orphan nuclear receptor (ligand unknown) and has a DNA-binding domain comprising two highly conserved zinc fingers.

During mouse embryogenesis, SF1 is expressed in the urogenital systems of both sexes from about day 9 after mating (Fig. 3a). SF1 is detected in the somatic cells of the coelomic epithelium that go on to become the Sertoli and interstitial cells (presumably Leydig cells) of the male and the granulosa cells in females (Ikeda et al., 1994; Shen et al., 1994; Schmahl et al., 2000). From day 12.5 after mating, expression in male gonads persists in both the Sertoli and Leydig cells, but its expression is downregulated in females from day 13.5 after mating (Ikeda et al., 1994). SF1 expression is not high in females again until late gestation, when it is localized to the thecal cells of the ovary.

SF1 positively regulates many genes involved in steroidogenesis, including genes encoding aromatase and 3β-hydroxysteroid dehydrogenase, which are involved in oestrogen and testosterone production by the gonadal steroidogenic cell lineages (for a review, see Parker and Schimmer, 1997). SF1 also regulates the expression of non-steroidal genes in the testis, such as AMH (see previous section). SF1 synergizes with WT1 and interacts with SOX9 to transactivate the AMH promoter in vitro (de Santa Barbara et al., 1998; Nachtigal et al., 1998). DAX1 can recruit the co-repressor N-CoR to SF1 in vitro, thus reducing its activity (Crawford et al., 1998).

Knockout mice carrying homozygous deletions of SF1 lack gonads and adrenals and die shortly after birth. In these mice, the gonads begin to form, are colonized by germ cells, but cease development at day 11.0–11.5 after mating and then undergo apoptosis (Luo et al., 1994). Therefore, it is thought that SF1 is important for creating an environment suitable for somatic cell proliferation, differentiation and maintenance, rather than for gonad formation and testis determination per se.
In all vertebrates examined to date, expression of SF1 is observed in the gonadal primordium before sex determination, supporting a role for SF1 in establishing a functional bipotential gonad. However, changes in the relative amounts of SF1 in male and female gonads during the sex determination period are remarkably variable among species. In the Red-eared slider turtle (Trachemys scripta), a species that uses temperature-dependent sex determination, SF1 expression is maintained in males and downregulated in females during the temperature-sensitive period (Fleming et al., 1999). However, in alligators, which also use temperature-dependent sex determination, SF1 expression is maintained in females and downregulated in males from stage 23, during the temperature-sensitive period (Fig. 3c; Western et al., 2000). In chickens, SF1 expression is maintained in males, but is upregulated in females during the period of sex determination (Fig. 3b; Smith et al., 1999a,b).

In chickens and alligators, high SF1 in the ovaries is probably correlated with the higher steroidogenic activity of the embryonic ovary compared with the testis in these species. Specifically, high SF1 in the ovary possibly reflects its involvement in the stimulation of aromatase expression, which is critical for normal ovarian development in chickens and alligators (Elbrecht and Smith, 1992; Lance and Bogart, 1992). In mice, aromatase is not expressed until late in ovarian development, so ovarian development is largely independent of oestrogen synthesis. Therefore, in mice, increased SF1 expression in the testis compared with the ovary presumably reflects its important role in Sertoli cell function in production of AMH and testosterone, respectively.

The AMH promoter has a consensus SF1 binding site in all species examined to date (de Santa Barbara et al., 1998; Oreal et al., 1998). SF1 has a role in upregulating Amh in mice, and SF1 expression in the male gonads of chickens and alligators during the period of sex determination may reflect a conserved role for it in the regulation of AMH. As SF1 is expressed before the onset of AMH expression in chickens and alligators, it may initiate AMH expression in these species. However, there must be other factors involved, as SF1 is expressed well before AMH is detected in both chickens and alligators. Furthermore, other factors must contribute to the expression of AMH in the male gonads of chickens and alligators, as SF1 expression is higher in females than males at the time when AMH expression is higher in males than females. SOX9 is an attractive candidate for regulating the expression of AMH in the male gonads of chickens and alligators. However, sexually dimorphic AMH expression precedes SOX9 expression in chickens and alligators, indicating that additional genes are probably required.

**Dosage sensitive sex reversal (DAX1)**

DAX1 (dosage sensitive sex-reversal-adrenal hypoplasia congenita-critical region of the X chromosome, gene 1) is an X-linked gene in humans, which when duplicated results in XY sex reversal. Mutations in DAX1 are responsible for adrenal hypoplasia congenita (AHC) in humans. DAX1 is a member of the orphan nuclear hormone receptor family, comprising a conserved C-terminal ligand-binding domain, but possessing three-and-a-half tandem copies of a 67 amino acid repeat, instead of the classical zinc-finger DNA binding domain.

During mouse embryogenesis, Dax1 expression is first detected from day 10.5 after mating throughout the gonadal primordium of both sexes (Fig. 3a). Dax1 expression is upregulated in both sexes from day 11.5 after mating and its expression is subsequently downregulated in males from day 12.5 after mating, but persists in females throughout ovarian development (Swain et al., 1996, 1998). Dax1 is expressed in the somatic cells of the bipotential gonad of both sexes. After sex determination, Dax1 is expressed in the Sertoli and Leydig cells of the testis and in the somatic cells of the ovary (Ikeda et al., 1996; Swain et al., 1996; Tamai et al., 1996). However, Dax1 expression is restricted to the testicular Leydig cells and ovarian thecal and granulosa cells in late embryonic development and during adulthood (Ikeda et al., 1996; Swain et al., 1996). The dynamic temporal and spatial expression of Dax1 within the male and female gonads during embryogenesis and beyond indicates that it may perform multiple functions.

Dax1 is proposed to be an antagonist of Sry function in males, as both genes are expressed in the same tissues and at the same time (Swain et al., 1998). Furthermore, co-expression of Dax1 and Sry from the Dax1 promoter in transgenic mice results in 100% XX females, whereas transgenic XX animals carrying only the Sry transgene develop as males (Swain et al., 1998). Dax1 is also expressed in the same tissues as Sf1, including the adrenal gland, gonad, hypothalamus and pituitary, indicating that they may act in the same developmental pathway (Ikeda et al., 1996). Several in vitro studies support a role for DAX1 protein in the inhibition of SF1-mediated transcriptional activation (Ito et al., 1997; Zazopoulos et al., 1997; Crawford et al., 1998; Nachtigal et al., 1998). These studies have proposed a number of mechanisms by which DAX1 and SF1 might function, so the exact nature of the DAX1–SF1 interaction in vivo remains unclear at present. It is unclear whether DAX1 exerts a repressive effect on SF1 due to direct protein–protein interactions, recruitment of co-repressors or by DNA binding. It is possible that distinct combinations or all of these functions may operate in vivo under different cellular and developmental conditions. To date, all DAX1 mutations detected in patients with ACH localize to the C-terminal part of the gene, so it is likely that this region represents the critical domain for DAX1 function in vivo.

Male mice overexpressing Dax1 can develop as females, but only in the presence of a late acting or ‘weak’ Sry allele (Swain et al., 1998). However, Dax1 is considered to be an ‘anti-testis’ gene, rather than a ovary-determining gene, as XX mice carrying homozygous deletions of Dax1 develop as normal females (Yu et al., 1998). These mice also display...
compromised endocrine function, indicating that Dax1 may play a more critical role in the endocrine function of the gonad, rather than in sex determination.

In chickens and alligators, the DAX1 C-terminal putative ligand-binding domain shows high amino acid identity with the same region in mammals (Smith et al., 2000; Western et al., 2000). However, chicken and alligator DAX1 lack the N-terminal tandem repeat regions containing the putative DNA binding domain in mammalian DAX1 (although chicken and alligator DAX1 amino acid sequences align quite well in this region). The lack of conservation of the N-terminal region might reflect a divergence in function of DAX1 between mammals compared with chickens and alligators.

In chicken gonads, RNase protection assays were used to show DAX1 expression at stage 28 in both sexes before sex determination (Fig. 3b; Smith et al., 2000). DAX1 expression increases between stage 28 and stage 30 in females, and continues to increase until at least stage 36. DAX1 expression also increases in male gonads after stage 30, but amounts are always markedly lower than in females and decrease significantly by stage 35, after the sex-determining period (Smith et al., 2000). In chickens, the sexually dimorphic expression of DAX1 is consistent with that observed in mice, but unlike the situation in mice, DAX1 is not downregulated in males at the onset of sexual differentiation, but is downregulated afterwards. RT–PCR on gonadal samples from alligator embryos raised at male- and female-producing temperatures showed that DAX1 was upregulated from stage 23, during the temperature-sensitive period, but was not sexually dimorphic during gonadogenesis (Fig. 3c; Western et al., 2000).

A role for DAX1 repressing testis development in chickens is consistent with its increased expression in the gonads of females compared with males during the sex-determining period, but this remains to be demonstrated. In alligators, the timing of DAX1 expression is consistent with a function in sex determination, but if DAX1 is performing an anti-testis function in alligators, other factors must also be involved, as DAX1 is not female-specific. The available evidence in mice suggests that Dax1 is inhibitory to Sf1 action. However, it is possible that in chickens and alligators, DAX1 performs the opposite function; it may augment SF1-mediated transcription of steroidalogenic genes such as aromatase and hence promote ovarian development. To date, the role of DAX1 in the regulation of steroidalogenic genes in chickens and alligators remains to be investigated.

**DMRT1**

DMRT1 (doublesex- and mab-3-related transcription factor 1) is the best conserved sex-determining gene in metazoans. DM-containing genes in Caenorhabditis elegans (mab-3) and Drosophila melanogaster (dsx) are also involved in differentiation of the gonads and encode transcription factors that are thought to bind DNA through their DM domains (Raymond et al., 1998).

In mice, Dmrt1 expression is detected by day 9.5 after mating (Fig. 3a). From day 10.5 after mating, Dmrt1 is expressed exclusively in the bipotential gonads of both sexes. From day 12.5 after mating, Dmrt1 transcripts are localized to the Sertoli and germ cells of the testis and in a punctate pattern in the ovaries (Raymond et al., 1999). By day 14.5 after mating, Dmrt1 expression is clearly higher in the testis compared with the ovary and by day 15.5 after mating, it is only weakly detected in the ovary.

Although it is clear that the expression of DMRT1 is gonad-specific, it has not been established whether it has a role in sex determination. Humans carrying deletions of chromosome 9 that include DMRT1 are XY sex-reversed (Veitia et al., 1997). However, at least two other DM-related genes map to the same region of chromosome 9 that is deleted in these sex-reversed individuals. Several studies have failed to find distinct mutations in the DMRT1 gene of humans with XY sex reversal. Mice carrying homozygous deletions of Dmrt1 are not XY sex-reversed, but they are infertile, due to severe testicular failure and defects in proliferation of germ cells (Raymond et al., 2000). To date, no upstream regulators or downstream targets of DMRT1 have been identified, making it impossible to place DMRT1 in any gene regulatory pathway.

In chickens, DMRT1 is located on the Z sex chromosome and its transcripts are detected from stage 19 (Raymond et al., 1999). DMRT1 is upregulated in male gonads compared with female gonads as early as stage 22, well before any morphological differentiation of the gonads (Fig. 3b; Raymond et al., 1999; Smith et al., 1999c; Smith and Sinclair, 2001; C. Smith and A. Sinclair, unpublished). The increased expression in males is greater than the twofold that would be expected from having two expressed copies and therefore its upregulation in males is likely to be specifically regulated.

In alligators, DMRT1 expression is detected from stage 20 in both sexes from the earliest stages of gonadogenesis (Fig. 3c; Smith et al., 1999c). DMRT1 expression increases between stage 23 and stage 24 in the gonads of both sexes, but is greater in the gonads of embryos incubated at male-producing temperatures. Hence, Dmrt1 represents a widely conserved male-upregulated gene.

In summary, although Dmrt1 is not likely to be involved in testis determination in mice, its chromosomal localization and tissue-, temporal- and sex-specific expression make it a strong candidate for a testis-determining factor in chickens. However, other genes must be required to initiate testis differentiation in males, as DMRT1 is expressed in both sexes well before the sex-determining period. Thus, DMRT1 is unlikely to represent an SRY-like ‘switch’ in chickens. The same is true in alligators, as DMRT1 is present in both sexes before the temperature-sensitive period and is upregulated in males only towards the end of the temperature-sensitive period.

**Conclusion**

Mice, chickens and alligators use distinct mechanisms to initiate the cascade of genes that determines the sexual fate
of the bipotential gonad. The genes described in this review represent some of the key sex-determining genes known in mice. These genes are also expressed during gonadogenesis in chickens and alligators, but the timing of expression relative to the critical sex-determining period, sequences and the sexual dimorphism of their expression vary considerably among the different species. This finding indicates that the interplay between these genes in mammalian sex determination might differ in other species. Despite this, morphological development of the testis and ovary is remarkably similar across these species.

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