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.

The existence of two sexes is nearly universal in the animal kingdom and although gonadal morphogenesis is remarkably similar across vertebrates, the sex-determining mechanism varies considerably. In most mammals, including humans, mice and marsupials, sexual fate is determined genetically by the presence of the *SRY* gene (sex-determining region on the Y chromosome; *Sry* in mice), which encodes the testis-determining factor on the Y chromosome (Sinclair *et al.*, 1990; Koopman *et al.*, 1991). Similarly, avian sex is determined genetically, although unlike mammals, the heterogametic sex in birds is female (ZW sex chromosomes), whereas males are homogametic (ZZ sex chromosomes). To date, it is not clear whether the sex-determining mechanism in birds is a dominant ovary-determining gene residing on the W chromosome or whether a double dose of a Z-linked gene triggers testis determination (for a review, see Clinton, 1998). The genetic switch in birds may involve both W- and Z-chromosome-based mechanisms. In contrast to mammals and birds, reptiles such as the American alligator do not have dimorphic sex chromosomes. Instead, this species uses temperature-dependent sex determination; all eggs incubated at 32.5–33.0°C during the sex-determining period become male and most eggs incubated outside these temperatures become female (Lang and Andrews, 1994). Hormones are also critical for sex determination in birds

and alligators. Ectopic administration of oestrogen or inhibitors of oestrogen synthesis during a critical period of gonadogenesis in chickens and alligators can feminize or masculinize the gonads, respectively, regardless of chromosomal or environmental cues (Lance and Bogart, 1991, 1992; Elbrecht and Smith, 1992; Vaillant *et al.*, 2001).

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

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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.

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 Southee, 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

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; *WTI*: 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; *DAXI*: dosage sensitive sex-reversal-adrenal hypoplasia congenita-critical region of the X chromosome, gene 1; *AMH*: anti-Müllerian hormone.

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). Although several of these genes are broadly conserved in sequence and sites of expression among vertebrates, there are differences in their sexual dimorphism, timing of expression and putative functional domains. These differences may impinge on the interconnectivity of the gene network and could underscore some of the biological differences in sex determination between vertebrates. In this review,

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 gonadogenesis 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 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 gonadogenesis 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 gonadogenesis 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* in activating 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 3 β -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 and Leydig 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 AHC 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 an 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 steroidogenic genes such as aromatase and hence promote ovarian development. To date, the role of *DAX1* in the regulation of steroidogenic 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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