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Review

Sry and the hesitant beginnings of male development

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Abstract

In mammals, *Sry* (*sex-determining region Y gene*) is the master regulator of male sex determination. The discovery of *Sry* in 1990 was expected to provide the key to unravelling the network of gene regulation underlying testis development. Intriguingly, no target gene of SRY protein has yet been discovered, and the mechanisms by which it mediates its developmental functions are still elusive. What is clear is that instead of the robust gene one might expect as the pillar of male sexual development, *Sry* function hangs by a thin thread, a situation that has profound biological, medical and evolutionary implications.

Keywords: *Sry*; Sex determination; Molecular mechanism; Evolution; Testis development; Brain gender

Introduction

Sex determination – the process by which one of two alternative pathways of development is engaged in the embryo – is essential for sexual reproduction and hence the survival of almost all metazoan species. Despite this, an immense variety of sex determination mechanisms is used in the animal kingdom (reviewed by Morrish and Sinclair, 2002; Crews, 2003; Matsuda, 2005), indicating a high degree of evolutionary plasticity of the sex determination pathway. While this undoubtedly reflects the different biological and environmental idiosyncrasies of the different species involved, one thing is clear: nature continues to tinker with sex determination even though most other developmental mechanisms have arrived at a more stable solution.

Sex determination is a binary switch mechanism that equates, in simple terms, to the molecular event that locks the embryonic gonads into their fate as testes or ovaries. Sexual differentiation, on the other hand, comprises the subsequent events required for correct gonadal development, and genesis of secondary characteristics that embody the male or female sexual phenotype. Even though sex-determining mechanisms vary a great deal, gonadal development, gonadal function, genital development, gamete production, genital form and function and other fundamental sexual characteristics follow more similar themes in disparate taxa. What is it about sex determination that is so vulnerable to evolutionary change? And how is it that diverse molecular switch mechanisms used in different taxa converge on developmental pathways of male or female development that themselves seem well conserved?

The discovery of *Sry*, the mammalian Y-chromosomal sex-determining gene, promised to answer these and many other questions relating to the genetics and developmental biology of sex determination (Sinclair et al., 1990; Gubbay et al., 1990). The simple transgenesis experiment of adding *Sry* function to an XX mouse to induce male phenotype confirmed the master switch role of this gene (Koopman et al., 1991). But since then, little else about this gene has proven to be simple or straightforward. The fact that *Sry* resides on the Y chromosome makes it vulnerable to degradation. As a result, it is minimally conserved and shows some functional flaws, surprising indeed for a gene on which survival and propagation of mammalian species so keenly depends.

We review here the molecular genetics and developmental biology of *Sry* and its role in mammalian sex determination. Although some pieces of the sex-determination jigsaw have been pieced together over the last 15 years (Fig. 1), basic issues of molecular mode of action and exact cellular roles of *Sry* in the early developing gonads remain obscure (Koopman et al.,

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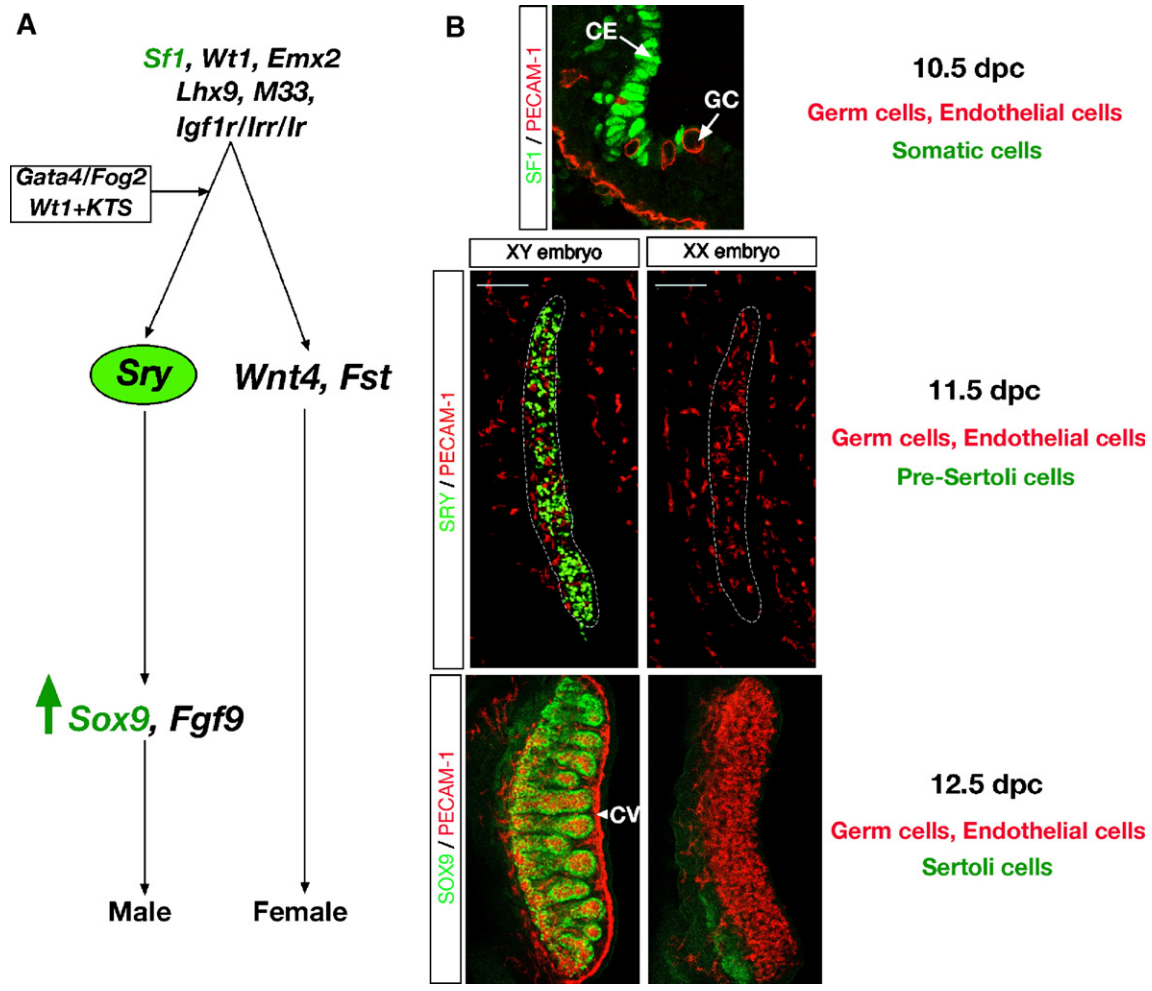

Fig. 1. Schematic representation of sex determination pathway in mammals. (A) Gene activities known to occur from 10.5 days *post coitum* (dpc) to 12.5 dpc, the critical period of sex determination. (B) Confocal gonadal images of gonad sections in the same period, immunostained with specific cellular markers. At 10.5 dpc both male and female gonads are seen as a thickening of the coelomic epithelium (CE), here shown as SF1-positive cells. From 10.5 to 11.5 dpc, several genes contribute to the development of the bipotential gonad, a stage marked by the migration of germ cells (GC) and proliferation of somatic cell lineages in both sexes. At this stage, Gata4/Fog2 and WT1+KTS are implicated in activation of *Sry* expression in the XY gonad. At 11.5 dpc, the morphology is the same for both male (left) and female (right) gonads, but male-specific markers such as *Sry*, and its downstream gene *Sox9* are already expressed. After the upregulation of *Sox9*, the morphology of the male gonad completely changes, as can be seen at 12.5 dpc by the presence of testis cords and the coelomic vessel (CV). In the absence of *Sry*, the female pathway takes place, involving *Wnt4* and *Fst* genes.

2001; Brennan and Capel, 2004). We show that *Sry* is a gene with intriguing quirks and weaknesses that likely explain its limited use as a sex determinant in the animal kingdom.

Regulation of Sry expression

Most of the functional information regarding *Sry* has been obtained using the mouse as a model system. In the mouse embryo, *Sry* exhibits a tightly-controlled and limited spatio-temporal profile of expression in the precursors of Sertoli cells of the XY gonad (Albrecht and Eicher, 2001; Sekido et al., 2004; Wilhelm et al., 2005). Early studies revealed that *Sry* is first expressed around 10.5 days *post coitum* (dpc), shortly after the emergence of the genital ridges, reaches peak levels of expression at 11.5 dpc, and is extinguished shortly after 12.5 dpc in mouse (Koopman et al., 1990; Hacker et al., 1995; Jeske et al., 1995). *Sry* mRNA is detectable only using sensitive methods such as reverse transcriptase-PCR and RNase protection, and appears to be expressed at lower levels than mRNAs of the related genes *Sox1*, −2 and −3 (Koopman et al., 1990; Hacker et al., 1995; Collignon et al., 1996).

Deeper analyses using *in situ* hybridization, transgenic reporter expression and immunofluorescence revealed that *Sry* mRNA and SRY protein display a dynamic expression pattern similar to a wave that emanates from the central longitudinal region of the genital ridges, then extends to rostral and caudal poles, and extinguishes in the order central–rostral–caudal (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Sekido et al., 2004; Wilhelm et al., 2005). Mouse SRY protein is robustly expressed at 11.5 dpc (Wilhelm et al., 2005; Taketo et al., 2005), despite the low level of mRNA expression. Individual cells are exposed to SRY activity for a period estimated at 8 h or less (Sekido et al., 2004; Wilhelm et al., 2005). SRY clearly throws a molecular switch to engage a male-

specific cascade of molecular events, but continued expression of SRY is not required for these events to unfold.

This distinctive expression profile raises a number of important questions. First, how is *Sry* transcription upregulated? Several factors expressed in the early genital ridges have been implicated in controlling expression of *Sry*, and an overview of how these factors probably interact to bring about male development is depicted in Fig. 2. Much attention has focused on Wilms' tumor-1 (WT1), mutations in which are associated with sex reversal in humans. WT1 has many variants, arguably the most significant of which are the +KTS and −KTS splice isoforms that include or exclude the amino acid sequence lysine–threonine–serine in the zinc finger nucleic acid binding domain. WT1−KTS and steroidogenic factor 1 (SF1) have been reported to bind and *trans*-activate the human and pig *Sry* promoter *in vitro* (Shimamura et al., 1997; de Santa Barbara et al., 2001; Hossain and Saunders, 2001; Pilon et al., 2003). The specific ablation of WT1−KTS produces smaller gonads, due to increased apoptosis, with very little differentiated tissue. Male specific markers such as *Sox9* and *MIS* were detected in a small cluster of cells, suggesting that WT1−KTS is not required for male sex determination (Hammes et al., 2001).

Conversely, mice lacking WT1+KTS show complete XY sex reversal due to reduction of *Sry* mRNA (Hammes et al., 2001). WT1+KTS does not transactivate the *Sry* promoter *in vitro* (Shimamura et al., 1997; Hossain and Saunders, 2001), and recently has been found to promote gene expression by enhancing mRNA translation (Bor et al., 2006). It is temping to speculate that WT1+KTS may act *via* effects on *Sry* mRNA translation (Fig. 2), which could in part explain differences between the low mRNA and robust protein expression observed for *Sry*. However, it is also possible that the 2-fold WT1−KTS overexpression that occurs in WT1+KTS null mice could be causing the observed sex reversal. Nevertheless, in view of these results, and because WT1 binding sites are present upstream of all mammalian *Sry* genes studied to date, regulation of *Sry* by WT1 is highly likely, but not proven *in vivo*. In particular it remains to be established whether reduced levels of *Sry* mRNA observed in WT1+KTS knockout mice are due to lower numbers of cells expressing *Sry*, or lower expression levels per cell.

Similarly, loss of function of several other genes by gene targeting in mice results in reduced *Sry* expression (Fig. 2). A remarkable triple knockout of the insulin receptor tyrosine kinase family genes *Igf1r*, *Irr*, and *Ir* demonstrated a requirement of these genes for the development of testes and showed a dramatic reduction in the expression of both *Sry* and *Sox9* (*S*RY-related HMG-b*ox* gene 9) (Nef et al., 2003). In mice in which *Gata4* and *Fog2* are either deleted or prevented from interacting, *Sry* expression is reduced also (Tevosian et al., 2002). In these cases, like the WT1+KTS knockout, it is not known whether the reduction is due to lower levels of *Sry* expression per cell or to fewer cells expressing *Sry*. Aside from these factors, it is evident that the ubiquitous transcription factor Sp1 is involved in *Sry* activation, because mutations affecting its binding to the *SRY* promoter have been associated with human XY sex reversal (Desclozeaux et al., 1998b; Assumpcao et al., 2005).

A second question of interest is how the duration of *Sry* expression is determined. Several lines of evidence point to the importance of 3′ untranslated region (UTR) sequences in *Sry* gene function and/or mRNA longevity. Hacker et al. (1995) first noted ATTTA mRNA destability sequences in the *Sry* 3′ UTR. On the other hand, *Sry* transgenes lacking the 3′UTR are unable to drive correct expression of reporter genes (Albrecht and Eicher, 2001; Sekido et al., 2004) or of eliciting male development (J. Bowles and PK, unpublished data). These observations suggest that the 3′UTR must contain essential functional information for *Sry* regulation, but the mechanisms and the 3′UTR specific sequence motifs involved have not been identified.

Associated with this question is the issue of how *Sry* expression is downregulated at 12.5 dpc, and whether this is

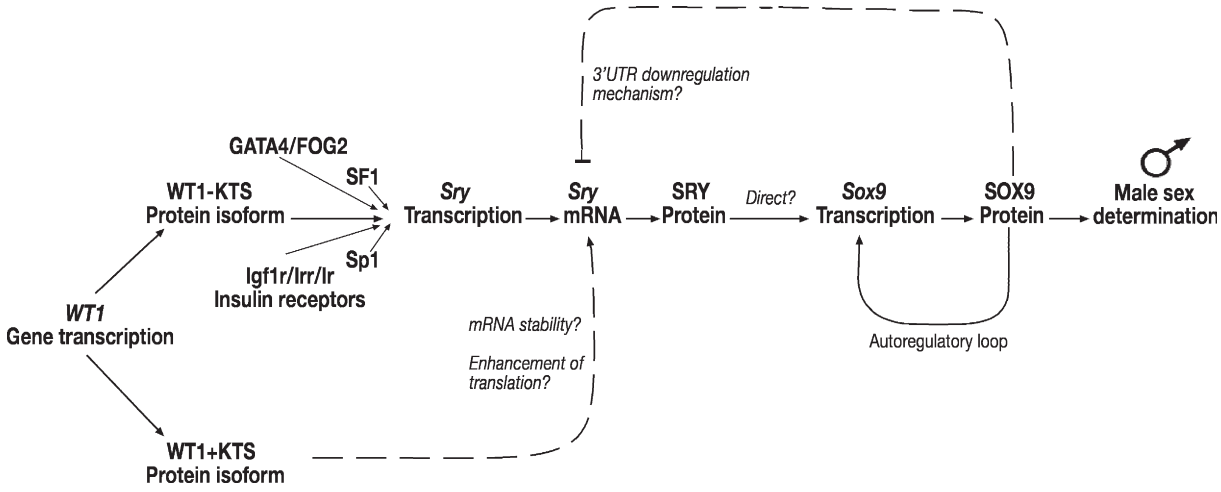

Fig. 2. Gene regulatory events involving SRY. Genetic interactions that establish the SRY-SOX9 axis during male sex determination. Several factors act in the supporting cell lineage to activate SRY, which subsequently triggers the upregulation of SOX9, either directly or indirectly, to lock-in the male sex determination pathway.

important for correct function. Strong evidence has emerged that SOX9 (see below) is the agent of *Sry* downregulation (Fig. 2): *Sox9* is upregulated shortly before *Sry* is downregulated, and in mice specifically lacking *Sox9* activity in fetal gonads, *Sry* expression persists (Chaboissier et al., 2004; Barrionuevo et al., 2006). The mechanism by which SOX9 shuts down *Sry* expression has not been clarified experimentally, but it is possible that *Sry* 3′UTR is involved, since, as mentioned above, it has been noted that *Sry*-promoter reporter transgene constructs lacking the 3′UTR are not downregulated at the correct time (Albrecht and Eicher, 2001; Sekido et al., 2004). Curiously, human *SRY* has a significantly shorter 3′UTR and is not downregulated, with expression persisting into adulthood in the gonads (Hanley et al., 2000). This could indicate that *SRY* has acquired an additional role beyond sex determination in humans, or alternatively, that its duration of expression has no functional relevance, being shorter in mouse with no consequences for *Sry* function. Consistent with the latter possibility, recent studies have revealed no noticeable functional consequences before or after the sex determination period in transgenic mice expressing *Sry* constitutively (Kidokoro et al., 2005).

Critical role of timing and level of Sry expression

Although the duration of *Sry* activity is evidently immaterial, both the time of onset and expression levels of *Sry* are decisive in male sex determination (Nagamine et al., 1999; Albrecht et al., 2003; Bullejos and Koopman, 2005). Because SRY is a transcription factor, it is hardly surprising that sufficient levels are required for proper function, particularly given the dosage-sensitivity that affects the function of other transcription factors in the SRY-related SOX family (Semba et al., 2000; Moreno-Mendoza et al., 2004; Qin and Bishop, 2005; Woods et al., 2005). Also, SRY lies at the fork of two alternative developmental pathways, and it might be predicted that a temporal window of opportunity needs to be seized to initiate testis development before onset of the ovarian determining pathway. What is surprising, however, is how barely SRY meets its threshold and timing requirements.

Evidence relating to this issue comes mostly from the study of unusual strain combinations in mice. Y chromosomes from natural populations of *Mus domesticus* captured in Val Poschiavo (Switzerland) and Tirano (Italy), termed Y^{POS} and Y^{TIR} respectively, are not able to elicit normal testis development when crossed onto a C57BL/6J background, leading to a variety of phenotypes from delayed testis development, through hermaphroditism with ovotestes, to complete XY sex reversal (Eicher et al., 1982). Analyses of the timing and levels of *Sry* expression in these cases have confirmed that *Sry* expression is certainly delayed and may also be reduced in these strain combinations (Nagamine et al., 1999; Albrecht et al., 2003; Bullejos and Koopman, 2005). In these cases, *Sox9* expression is also delayed and, in ovotestes, becomes confined to the central portion of the gonad only (Moreno-Mendoza et al., 2004; Bullejos and Koopman, 2005). The position of the *Sox9* expression domain and hence of the testicular material in these ovotestes most likely reflects the dynamic wave of *Sry* expression (Albrecht et al., 2003; Bullejos and Koopman, 2005). In addition, SRY protein isoform differences between the two strains involved may exacerbate the genetic background dependence of the degree of sex reversal (Lee and Taketo, 2001), by affecting interactions of SRY with autosomal genes required for sex determination (Coward et al., 1994).

From these studies it can be concluded that threshold levels of SRY must be achieved in individual cells during a critical time window to appropriately activate *Sox9* and thus trigger testis determination before the ovarian determining pathway engages (Fig. 3A; Palmer and Burgoyne, 1991; Bullejos and Koopman, 2005). Failure to reach this threshold within the required time window to activate the trigger appropriately, due to subtle genetic background variations, may be an unrecognized contributor to cases of human XY sex reversal for which no mutations in SRY or other genes have been detected.

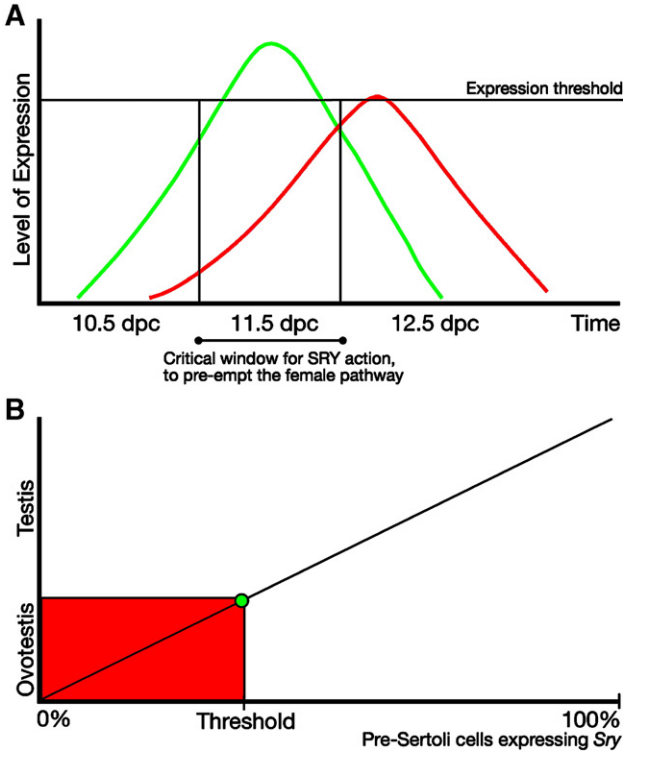

Fig. 3. Thresholds of SRY expression and Sertoli cell numbers are essential for proper testis determination. (A) The level of expression of *SRY* (green curve) within individual Sertoli cell precursors must reach a threshold level within a critical time window, required for the complete engagement of downstream molecular machinery, in particular the upregulation of *SOX9*. The Y^{POS} *Sry* allele (red curve) is unable to function properly in C57BL/6J mouse background, evidently because it does not reach the expression threshold within the critical time window. (B) A threshold number of Sertoli cells (green dot) has to be reached to allow the appropriate assembly of testis cords. The minimal percent of cells compatible with cord formation was revealed in XX↔XY chimera experiments, but most likely reflects the proportion of Sertoli cells expressing robust levels of *Sry* that has to be reached in normal XY gonads. In both situations, below the threshold number an ovotestis is formed, or an ovary when pre-Sertoli cell numbers are near or equal to zero. We postulate that Prostaglandin-D_2-mediated paracrine signaling recruits somatic cells into the Sertoli cell pathway, so that the threshold of Sertoli cells is reached in situations where *Sry* expression is compromised.

Cellular roles of Sry

The emerging picture of the cellular roles of *Sry* reinforces the precarious nature of its ability to make males. The primary function of *Sry* is to induce Sertoli cell differentiation. More correctly, *Sry* induces differentiation of pre-Sertoli cells, which become Sertoli cells when they assemble into testis cords (Fig. 1B). Pre-Sertoli cells arise from a bipotential lineage known as supporting cell precursors, on account of the role of Sertoli cells and their ovarian counterpart, granulosa cells, in supporting the growth and maturation of germ cells (Albrecht and Eicher, 2001).

Pre-Sertoli and Sertoli cells are thought to orchestrate the organogenesis of the testis in a number of ways (Fig. 4), but it is not clear which roles are mediated by *Sry* itself, and which are indirect downstream effects of *Sry* action. That *Sry* is active in the supporting cells was long predicted because pre-Sertoli cells are the first cell type to differentiate in the gonad. Also, classic embryological studies showed that in XX↔XY chimeras, almost all Sertoli cells are XY whereas other cell types are a mixture of XX and XY cells, suggesting that the testis-determining gene acts cell-autonomously to induce differentiation of (most) pre-Sertoli cells, and that these signal to bipotential precursors of other cell types to induce their male-type differentiation (Palmer and Burgoyne, 1991). This role of *Sry* in Sertoli cell development was confirmed using a transgenic reporter approach to trace the fate of *Sry*-expressing cells in the mouse gonad (Albrecht and Eicher, 2001; Sekido et al., 2004).

In the XX↔XY chimera experiments of Burgoyne and Palmer (1991, 1993), a small proportion (up to 10%) of Sertoli cells were always detected that were XX, and so lacking *Sry*. These authors interpreted their observations as indicating the existence of a paracrine pathway downstream of *Sry*, involved in recruiting non-*Sry*-expressing XX supporting cells into the Sertoli pathway (Palmer and Burgoyne, 1991). Recent studies have revealed the nature of this recruitment pathway. Prostaglandin D_2 (PGD_2), a product of the early testis, is able to induce testicular development of XX genital ridges in culture (Adams and McLaren, 2002). Wilhelm et al. (2005) showed, in XX↔XY gonadal cell mixing experiments, that PGD_2 is sufficient to allow XY cells to recruit XX supporting cells to differentiate as pre-Sertoli cells, *via* upregulation of *Sox9* expression. Moreover, PGD_2 has also been implicated in SOX9 nuclear translocation (Malki et al., 2005), a step necessary for subsequent pre-Sertoli cell differentiation. It has been postulated that in cells where pre-Sertoli cell differentiation is not induced through cell-autonomous action of SRY, recruitment by neighboring SRY-positive cells might serve to maximize the number of supporting cells that develop as pre-Sertoli cells (Fig. 4). In this hypothesis, PGD_2 provides a canalization mechanism to ensure testicular development in situations where SRY expression is weak or its function impaired (Wilhelm et al., 2005).

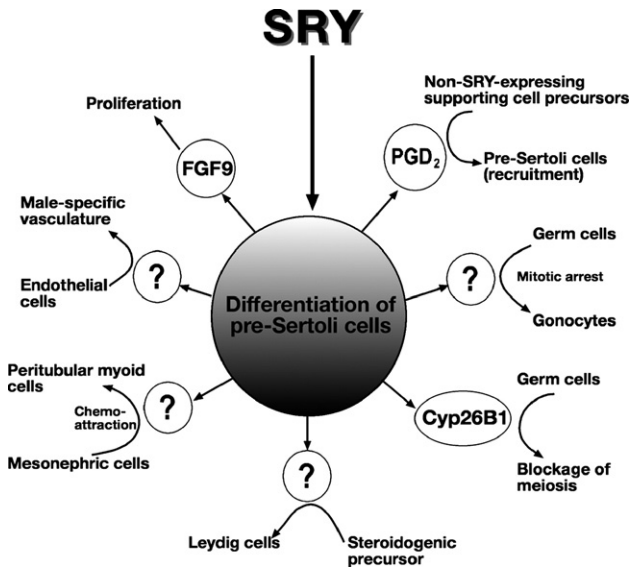

Fig. 4. Cellular events surrounding pre-Sertoli cell differentiation. The complex cascade of cellular events following Sertoli cell differentiation includes signaling to other cell lineages (reviewed by Ross and Capel, 2005). It is possible that some or all of these events might be mediated by SOX9 and not by SRY itself.

If this hypothesis is correct, it implies not only that SRY expression needs to reach threshold levels to induce differentiation of individual pre-Sertoli cells, but also that threshold numbers of pre-Sertoli cells need to be achieved to induce complete testis development. XX↔XY chimera experiments have suggested that approximately 20% of pre-Sertoli cell precursors need to be XY and thus express *Sry*, in order to initiate and reinforce testis formation. If numbers of XY (*Sry*-expressing) supporting cells fall below that threshold, ovarian (or ovotestis) differentiation is initiated (Fig. 3B; Palmer and Burgoyne, 1991; Burgoyne and Palmer, 1993). This threshold population concept is supported by investigations on the role of proliferation in sex determination. Using proliferation inhibitors both *in vivo* and in gonad culture, it was possible to demonstrate that an eight-h period of early proliferation is necessary for the differentiation and production of a population that contains Sertoli cell precursors (Schmahl et al., 2000; Schmahl and Capel, 2003). This period coincides with the expression of *Sry*, and may be part of a mechanism to increase the number of Sertoli cell precursors above the threshold necessary to direct testis determination (Schmahl et al., 2000; Schmahl and Capel, 2003). Consistent with this scenario, loss of function of the proliferation factor FGF9 produces XY sex reversal (Colvin et al., 2001).

Migration of cells from the mesonephros into the developing gonad is a further crucial event in testis formation that depends on *Sry* activity (Fig. 4). The immigrant cells mostly consist of endothelial cells, but include some interstitial cells and peritubular myoid cells, which co-operate with pre-Sertoli cells to generate testis cords (Buehr et al., 1993; Martineau et al., 1997; Capel et al., 1999). Immigration of mesonephric cells is required during a specific time window of gonad development (Tilmann and Capel, 1999) and, if blocked, prevents cord formation and differentiation. Sandwich culture experiments indicated that XY gonads can induce mesonephric cell migration through an interposed XX gonad, indicating that chemoattractive signals are involved.

In summary, an induced burst of pre-Sertoli cell proliferation is evidently important to ensure that enough of these cells are produced to orchestrate further testis development. If necessary, numbers of pre-Sertoli cells can be further augmented by PGD_2-mediated recruitment. The rapid requirement for cells of other lineages is satisfied in part by chemoattraction of cells from the mesonephros. The key unanswered question in evaluating the cellular role(s) of *Sry* is whether these events are direct effects of *Sry* or are mediated by downstream genes such as *Sox9*, a gene that has been shown to be sufficient for testis determination in transgenic mice (Vidal et al., 2001). The overlapping expression patterns of *Sry* and *Sox9* make it difficult to discriminate between the cellular roles of each gene. An answer to these questions awaits analysis of mice that lack *Sry* but overexpress a *Sox9* transgene (for example, Vidal et al., 2001), and *Sox9* knock-out mice that retain *Sry* but lack *Sox9* (Chaboissier et al., 2004; Barrionuevo et al., 2006).

Molecular properties of the SRY protein

Available evidence suggests that *Sry* must be expressed in the right place (supporting cell precursors), at the right time (10.5–11.5 dpc) and with appropriate levels of expression to allow for proper male sex determination. How can these constraints be explained at a molecular level? What are the molecular consequences of *Sry* action, and how are these accomplished? To address these questions, we turn to the biochemical properties of the SRY protein.

SRY is a member of the SOX family of developmental transcription factors (Bowles et al., 2000; Schepers et al., 2002). Like other SOX proteins, SRY's signature sequence is a 79 amino-acid motif known as the HMG domain (Fig. 5), that enables it to bind to the DNA consensus sequence (A/T)ACAA (T/A) with high affinity (Harley et al., 1994). Human and mouse SRY binds DNA in the minor groove, causing it to bend at angles of 60–85° in both species (Giese et al., 1994; Werner et al., 1995). These properties are shared with other HMG domain-containing factors such as LEF1, which acts as an architectural factor that facilitates interaction between widely separated protein binding sites and promotes the assembly of transcriptional complexes (Giese et al., 1992).

Most *SRY* mutations that cause male to female sex reversal in humans affect the ability of SRY to bind and bend DNA (Fig. 5A; Harley et al., 1992; Jager et al., 1992; Pontiggia et al., 1994; Schmitt-Ney et al., 1995; Mitchell and Harley, 2002). In the mutation M61I, the DNA binding affinity of the mutated SRY was similar to wild-type SRY, but DNA bending was affected (Pontiggia et al., 1994), suggesting that DNA bending is necessary for SRY function, and that the bend angle might be critical. However, the essential role of the bend angle can be questioned in the light that human and goat SRY can cause XX sex reversal in transgenic mice (Lovell-Badge et al., 2002; Pannetier et al., 2006), despite the differences in bending ability mentioned-above for human and mouse SRY. In addition, a recent reassessment of the M64I mutation revealed that the DNA-bending defect was an inadvertent consequence of protein truncation *in vitro*, and can be rescued by restoring the truncated

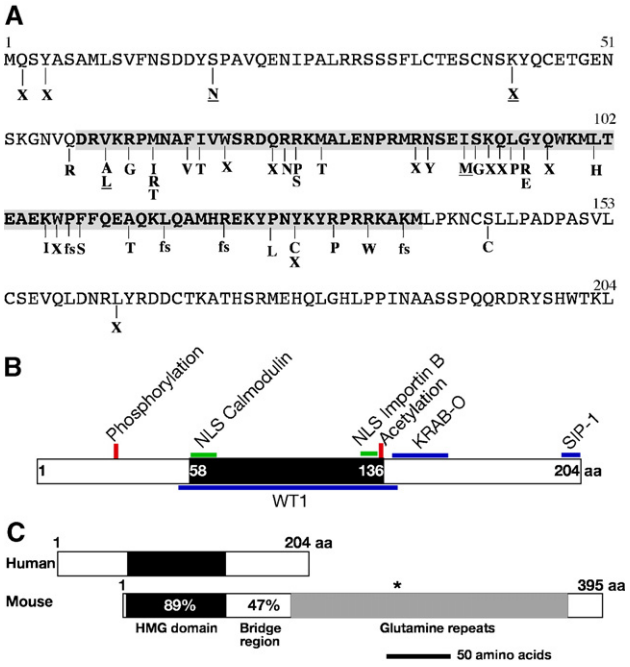

Fig. 5. Structure of the SRY protein. (A) Human SRY is a 204 aa protein containing a central HMG domain (shaded and bold). Missense mutations causing XY gonadal dysgenesis are shown below the sequence (references for mutations are given in the text). Familial mutations are underlined. fs, frameshift; X, stop codon. (B) Human SRY protein diagram showing the mapping of reported interactions. The HMG domain is represented as a solid box flanked by the N-terminal and C-terminal domains. Numbers indicate amino acid residues. Nuclear localization signals (NLS) interacting with calmodulin (N-NLS) or importin β (C-NLS) are shown in green. Post-translational modifications regulating SRY activity are shown in red, as well as reported stable interactions with nuclear proteins in blue. (C) Comparison of human and mouse SRY proteins. Level of amino acid homology to human SRY in the HMG box and C-terminal region of the proteins are shown. The mouse protein contains a large glutamine-rich repetitive region shown as a shaded box. SRY in the subspecies *M. m. domesticus* has a premature stop codon (asterisk) that truncates this domain. Apart from the HMG domain, the bridge region in mouse (47% homology to the complete C-terminus of human SRY) is the only region homologous to human SRY. Sequences were sourced from GenBank, accession numbers L08063 (human) and U70655 (*Mus musculus musculus*, mouse). Percent homologies at the amino acid level were calculated using the ClustalW algorithm.

basic C-terminus of SRY (Li et al., 2006). This study also revealed that the SRY C-terminal domain contributes to SRY conformation, and that fluctuations in its conformation can affect SRY function. In brief, the available evidence does not conclusively demonstrate whether SRY acts as a conventional transcription factor, or an architectural transcription factor, or both.

To further confound the issue, SRY has been implicated in pre-mRNA splicing (Ohe et al., 2002; Lalli et al., 2003). However, the direct relevance of this observation for sex determination *in vivo* has not been established, since no mRNA essential for sex determination has been shown to specifically require SRY for its correct splicing. In addition, the experiments reported by Ohe et al. (2002) showed that SRY, SOX6 and SOX9 are redundant in pre-mRNA splicing, and that only their HMG domains are necessary for the splicing function (Ohe et al., 2002). Therefore, it may be that this is a vestige of shared ancestry with other members of the HMG superfamily.

Apart from DNA binding and bending, the SRY protein is involved in several protein–protein interactions that determine its nuclear localization as well as its likely target gene specificity (Fig. 5B; Reviewed by Wilson and Koopman, 2002; Smith and Koopman, 2004). Like other SOX factors (Kamachi et al., 1999, 2000; Wilson and Koopman, 2002), SRY almost certainly needs to interact with partner factors to achieve an active protein complex and to provide the ability to discriminate between targets with similar binding sites. Moreover, in a transgenic mouse line constitutively expressing *Sry*, the onset of *Sox9* expression occurs in the same temporal wave in the genital ridges as in wild-type embryos, suggesting that activation of SRY target genes is dependent on a protein partner that is itself upregulated in a wave pattern (Kidokoro et al., 2005), although the possibility of translational and post-translational regulation cannot be ruled out.

Several protein–protein interactions have been reported to modulate SRY activity (Fig. 5B). The SRY-interacting PDZ protein SIP-1 could function as a module mediating protein–protein interactions between SRY and other transcription factors, a function conserved between human and mouse SRY (Poulat et al., 1997; Thevenet et al., 2005). Interestingly, the same "bridge" region of mouse SRY involved in the interaction with SIP-1 also directly interacts with a novel protein, KRAB-O, containing only a Krüppel-associated box domain (Oh et al., 2005), however in human SRY the binding motifs for SIP-1 and KRAB-O are separated (Fig. 5B). By means of this interaction with KRAB-O, SRY might regulate its yet unidentified downstream target genes by recruiting a silencing complex that contains KAP1 and HP1 (Oh et al., 2005). Furthermore, SRY synergizes with WT1, an interaction that might allow SRY to utilize the *trans*-activation domain of WT1 to activate promoters containing SRY-binding sites (Matsuzawa-Watanabe et al., 2003). Nevertheless, there is no *in vivo* loss- or gain-of-function evidence that proves or disproves any of these SRY nuclear interactions to be essential for sex determination, although it may be significant that mutations in the SIP-1/KRAB-O interaction motifs have been associated with human XY sex reversal (Fig. 5A; Tajima et al., 1994; Shahid et al., 2004).

A nuclear localization signal (NLS) at the C-terminus of the SRY HMG domain is essential for the proper nuclear import of SRY through the interaction with importin β (Forwood et al., 2001); SRY mutations in this NLS result in a reduction in nuclear import and likely explain some cases of human sex reversal (Fig. 5B; Li et al., 2001; Harley et al., 2003; Smith and Koopman, 2004). Acetylation further contributes to the nuclear localization of SRY by increasing SRY interaction with importin β, while specific deacetylation by histone-deacetylase-3 induces a cytoplasmic localization of SRY (Thevenet et al., 2004), indicating that acetylation and deacetylation could be regulating SRY action during early phases of testis determination. In addition, an N-terminal NLS import pathway appears to involve interaction of SRY with calmodulin (Harley et al., 1996, 2003; Kelly et al., 2003). However, the SRY M64I mutation impairs nuclear localization but does not perturb binding of SRY to calmodulin or the structure of the calmodulin–SRY complex (Li et al., 2006), and so it is not clear whether calmodulin or other interacting proteins mediate the function of the N-terminal NLS *in vivo*.

One characteristic that has complicated the analysis of SRY protein function is its high degree of variability in size and amino acid sequence among mammalian species, particularly at the C-terminus (Fig. 5C). This variability underscores the high rate of evolution of *Sry* (Tucker and Lundrigan, 1993; Whitfield et al., 1993; Pamilo and O'Neill, 1997; Katoh and Miyata, 1999). Such a remarkably high rate of evolution could be explained in two different ways: either the C-terminal domain of SRY is not functionally constrained, or it is undergoing a species-specific adaptive divergence by a process of positive Darwinian selection (Tucker and Lundrigan, 1993; Whitfield et al., 1993). Truncated SRY proteins were unable to cause XX sex reversal in transgenic mice, suggesting that the SRY C-terminal domain is important for sex determination (Bowles et al., 1999), although it is possible that protein or conformational instability could account for the observations. Conversely it has been reported that human *SRY* can cause sex reversal of XX transgenic mice, leading to speculation that the mouse C-terminus is not functional, because this domain is absent from human SRY (Lovell-Badge et al., 2002). The level of homology between the bridge region of mouse SRY and the C-terminus of human SRY (Fig. 5C), along with evidence showing that SIP-1 and KRAB-O interact with both SRY homologues (Fig. 5B), signify that these two regions are phylogenetically and functionally equivalent. Therefore, it is likely that both the HMG domain and the bridge domain are required for SRY function, but that the putative protein interaction function of the bridge domain is less constrained by sequence than the HMG domain. In addition, it is likely that human SRY C-terminus and mouse SRY bridge domain might be evolutionarily constrained at the three-dimensional structural level, as recent evidence shows that this region contributes to SRY conformation and function (Li et al., 2006).

The most likely function of the SRY C-terminus is transcriptional *trans*-activation or -repression. *In vitro* biochemical assays indicate that SRY is a transcriptional activator: mouse SRY activated transcription of a reporter gene containing multiple copies of the AACAAT binding site (Dubin and Ostrer, 1994). On the other hand, analysis of more than 100 cases of XX males has supported a model in which *SRY* might act by repressing a negative regulator of male sex determination; the logic underpinning this model is that *SRY*-negative XX maleness occurs at too high a frequency to be explained by gain-of-function mutation in a male sex-determining gene, but rather is more consistent with a loss-of-function mutation in a suppressor of maleness (McElreavey et al., 1993). Cases of familial XX sex reversal in which the parents did not exhibit the sex reversed phenotype, further support the notion of a recessive allele acting as a repressor of male sex determination (McElreavey et al., 1993). Indeed, *in vitro* experiments showed that SRY can act as a repressor depending on its phosphorylation status (Desclozeaux et al., 1998a). It is also plausible that SRY might act as a repressor that provokes local heterochromatinization of its target genes by interacting with a KRAB-only protein, as described above (Oh et al., 2005). In summary, it has

not been possible to demonstrate conclusively whether SRY acts as a transcriptional activator, repressor or architectural protein. Resolving this issue hinges on the identification of *in vivo* targets of SRY.

Is Sox9 a direct target of SRY?

Sox9 overexpression has been shown to be sufficient for testis determination in transgenic mice (Bishop et al., 2000; Vidal et al., 2001; Qin and Bishop, 2005), and *SOX9* duplication has been linked to human XX sex reversal (Huang et al., 1999). Furthermore, *SOX9* mutations result in XY sex reversal in the skeletal dysgenesis syndrome campomelic dysplasia (Tommerup et al., 1993; Foster et al., 1994; Wagner et al., 1994). Thus, *Sox9* is clearly a critical downstream gene from *Sry* in the male sex-determining pathway. But is *Sox9* a direct target of SRY action?

Sox9 transcription is upregulated barely 4 h after the onset of mouse *Sry* expression, and consequently both genes exhibit an overlapping expression pattern in developing pre-Sertoli cells (Kent et al., 1996; Morais da Silva et al., 1996; Sekido et al., 2004; Bouma et al., 2005; Bullejos and Koopman, 2005; Kidokoro et al., 2005; Wilhelm et al., 2005). Upregulation of *Sox9* by human *SRY* in a rat cellular system has suggested that SRY acts specifically on *Sox9*, without affecting other *Sox* genes, and that clinical mutations in SRY can impair this upregulation (Li et al., 2006). Available evidence has led to the widely held belief that *Sox9* is a direct target of *Sry* (Kent et al., 1996; Canning and Lovell-Badge, 2002; Sekido et al., 2004). Certainly, direct regulation of *Sox9* by SRY is the simplest scenario, and one that has not been experimentally discredited.

However, it is also possible that SRY may be able to activate *Sox9* indirectly. There are several instances in which *Sox9* can be upregulated in the absence of *Sry* both in the developing gonad and postnatally. As mentioned above, prostaglandin D_2 signaling can upregulate *Sox9* in XX developing gonads (Adams and McLaren, 2002; Wilhelm et al., 2005). Targeted deletion of either *Foxl2* or the estrogen receptor genes leads to postnatal upregulation of *Sox9* in XX gonads and *trans*-differentiation of granulosa cells into Sertoli cells (Couse et al., 1999; Ottolenghi et al., 2005). These observations suggest that the sex determination lock can be opened in the mammalian differentiated female gonad by means of the upregulation of *Sox9*, which is perhaps a remnant of non-mammalian environmental sex determination systems with more labile and permissive mechanisms (Crews, 2003; Matsuda, 2005). In non-mammalian species, which lack *Sry*, *Sox9* clearly can be activated by other means. Therefore, if several genes can take over the control of *Sox9* upregulation, it is formally possible that these may intervene between *Sry* and *Sox9*, a necessary intervention if SRY acts by negatively regulating a repressor of maleness (McElreavey et al., 1993). Hence, the regulatory relationship between *Sry* and *Sox9* remains a subject of considerable debate, and is arguably the million-dollar question relating to how SRY acts to bring about male sex determination.

Because *Sox9* function is widely conserved among vertebrates, and possibly even metazoans (DeFalco et al., 2003), and *Sry* is found only in mammals, it may be that *Sox9* represents the common master regulator of male sex and that the only function of *Sry* in mammals is to upregulate *Sox9* expression. Transgenic mice lacking *Sry* but overexpressing *Sox9* in the genital ridges appear to develop as normal males (Bishop et al., 2000; Vidal et al., 2001; Qin and Bishop, 2005), further implying that SOX9 is capable of activating the entire male pathway and that the function of SRY is limited to directly or indirectly causing upregulation of *Sox9*.

Sry beyond the gonads

In addition to a key role in testis determination, recent studies point to a direct role for SRY in sex-specific differentiation of the brain. *Sry* expression in the brain was reported some years ago (Lahr et al., 1995; Mayer et al., 1998, 2000), but its functional relevance has remained unclear. It has become axiomatic that sex differences in brain function are due to effects of gonadal hormones: testosterone clearly induces masculine differentiation and behavioral programming of the brain (Phoenix et al., 1959; MacLusky and Naftolin, 1981). Nevertheless, there is evidence suggesting that sex differences can be caused by cell-autonomous action of sex chromosome genes in mammals and birds (Arnold and Burgoyne, 2004).

Recently, Dewing et al. (2006) determined that SRY protein is expressed in the same brain cells that produce tyrosine hydroxylase, which is necessary for the synthesis of the neurotransmitter dopamine. Experimental downregulation of *Sry*, by means of unilateral microinfusion of antisense oligodeoxynucleotides in rat brains, showed that SRY is crucial for dopamine-secreting cells to function normally in motor control (Dewing et al., 2006). The paradox is that females, which lack *Sry*, do not exhibit an observable motor dysfunction; Dewing et al. suggest that *Sry* might compensate in males for a lack of estrogen, which in females influences tyrosine hydroxylase expression. Regardless, it is certainly interesting to consider that SRY may directly control other aspects of male-specific brain development and probably behavior (De Vries et al., 2002).

Concluding remarks

Sry is uniquely important in mammalian biology, lying at the crossroads of male and female development in the embryo. Despite this, *Sry* has been slow to yield its secrets, largely because it is poorly conserved and expressed in a small group of embryonic cells for a short interval of time. Experimentally, it has not been possible to pinpoint its regulatory sequences, or positively identify SRY protein partners required for *in vivo* function, and its most likely regulatory target, *Sox9*, is a closely related gene with an almost impossibly long upstream regulatory region (Wunderle et al., 1998; Pfeifer et al., 1999).

The picture that has emerged is one of a frail gene and protein with a tenuous grip on its important regulatory role, at least in mice. Clearly SRY must act within a limited window of time to enact the male pathway of gonadal development, and must exceed a threshold level of expression within this interval. While the precarious nature of such a system is at first glance

surprising, it is most likely a consequence of the location of *Sry* on the Y chromosome, a location in which it is subject to evolutionary decay without the recombination-based repair mechanisms available to X-linked and autosomal genes. It has been predicted that Y-chromosome will completely disappear within 10 million years, with the consequent loss of the *Sry* locus, implying that a new switch mechanism will need to arise within that time to supplant *Sry* function (Graves, 2002a). In fact, it has been proposed that this may already have occurred in some species of eutherian mammals that lack *Sry* (Just et al., 1995; Graves, 2002a,b).

Several new tools have become available for deciphering SRY function. Novel antibodies to mouse SRY will be useful to study endogenous SRY protein (Wilhelm et al., 2005; Taketo et al., 2005), as well as to find its target genes by chromatin immunoprecipitation. Microarray-based expression screens of male and female genital ridges (Bullejos et al., 2001; Smith et al., 2003; Boyer et al., 2004; Nef et al., 2005; Small et al., 2005; Beverdam and Koopman, 2006) will likely allow identification of potential regulators, interactors and targets of SRY. The availability of *Sox9*-overexpressing and *Sox9*-knockout mice will allow the molecular and cellular roles exerted by *Sry* and *Sox9* to be disentangled and studied independently (Vidal et al., 2001; Chaboissier et al., 2004; Akiyama et al., 2004; Barrionuevo et al., 2006), and the generation of further novel loss-of-function and transgenic lines will provide more of the missing pieces to the puzzle of events surrounding *Sry* action.

Clearly, *Sry* engages a complex and rapid cascade of molecular and cellular reorganization in the nascent testis. Precise timing is essential to ensure the complete success of the Sertoli cell program initiated by *Sry* and to counteract the opposing forces of the female sex-determining pathway. Further study of the role of *Sry* will answer fundamental remaining questions regarding how male sex is determined, and may provide a basis for diagnosis and management of intersex and sex reversal conditions in humans. Despite hesitant beginnings, the coming years are likely to yield important insights into the part played by *Sry* in the journey of male development.

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