Sry: the master switch in mammalian sex determination
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Summary
SRY, the mammalian Y-chromosomal testis-determining gene, induces male sex determination. Recent studies in mice reveal that the major role of SRY is to achieve sufficient expression of the related gene Sox9, in order to induce Sertoli cell differentiation, which in turn drives testis formation. Here, we discuss the cascade of events triggered by SRY and the mechanisms that reinforce the differentiation of the testes in males while actively inhibiting ovarian development.

Key words: Sry, Sex determination, Sox9, Testis, Sertoli cell

Introduction
The development of two sexes is observed in most animals and is essential for their survival and evolution. Disorders of sex development (DSDs; see Glossary, Box 1) are among the most common genetic diseases in humans and are often associated with genital ambiguity (Kronenberg and Williams, 2007). Because of its clinical and biological importance, identifying the mechanism of sex determination – the developmental decision to generate either testes or ovaries – continues to attract the attention of a broad range of researchers, including developmental biologists, biomedical scientists, evolutionary biologists and ecologists.

In mammals, two major breakthroughs have shaped our current understanding of sex determination. First, in 1959, two human DSDs, Turner syndrome (XO females) and Klinefelter syndrome (XXY males) were identified and reported (Ford et al., 1959; Jacobs and Strong, 1959), and these studies established that the Y chromosome carries a gene that determines maleness. It would take another 30 years before the second breakthrough was made: the discovery of SRY (sex-determining region on the chromosome Y), denoted Sry in species other than humans. The human SRY gene was identified by searching for conserved sequences among translocated Y chromosomal DNA from four XX male patients (Sinclair et al., 1990). The presence of a similar gene, Sox9, on the mouse Y chromosome was consistent with this gene having a sex-determining function (Gubbay et al., 1990). The role of Sry as the switch gene for mammalian sex determination was confirmed in experiments in which XX mice were converted to males by the introduction of Sry (Koopman et al., 1991).

Sry and the molecular mechanisms of sex determination have continued to be studied intensely over the past 20 years. Unlike other developmental systems that are well conserved through evolution, sex determination is highly variable in the animal kingdom, and the genetic mechanisms involved in common laboratory model organisms, such as flies, nematode worms, chickens and frogs, bear little, if any, resemblance to those used in mammals. Indeed, Sry is found only in mammals, though not in all mammalian orders – monotremes (see Glossary, Box 1), for example, lack Sry. Most of our current understanding of Sry and

Box 1. Glossary

Cell-autonomous. Occurring within a cell, not involving signalling between cells.
Chromatin immunoprecipitation (ChIP). A method used to identify the transcriptional targets of a given transaction factor by precipitating the transcription factor while it is bound to DNA, then characterizing the bound DNA.
Coelomic epithelium. Layer of cells lining the body cavity of an embryo.
Disorder of sex development (DSD). Any one of a spectrum of conditions where the development of internal or external sexual organs differs from ‘typical’ male or female, or is not as expected given the sex chromosomes present.
Eutherian mammals. A subclass of mammals that have a placenta.
Genital ridges. Pair of thickened rows of coelomic epithelial cells either side of the midline in the trunk of an embryo that are the precursors of the gonads.
Granulosa cells. The ‘nurse’ cells in ovarian follicles that nurture germ cells.
High-mobility group. A specific family of transcription factors that have structurally related DNA-binding domains ~80 amino acids long.
Leydig cells. Cells in the interstitium of testes that produce androgens.
Mesonephros. Embryonic structure attached to each genital ridge, from which the male or female internal sexual ducts arise.
Monotremes. A subclass of mammals, represented by platypus, that lay eggs instead of giving birth to live young.
Nuclear localization signal. A short sequence of amino acids that allows proteins such as transcription factors to move from the cell cytoplasm into the cell nucleus.
Ovotestis. A gonad containing both ovarian and testicular tissue.
Paracrine signalling. Short-range chemical communication between cells.
Pre-Sertoli cells. Cells in an XY genital ridge that have activated Sry and Sox9 expression, but have not yet assembled into testis cord structures.
Sertoli cells. Testicular cells that form testis cords and interact with and nurture germ cells.
Testis cord. The precursors of the adult spermatogenic tubules, composed of germ cells enclosed by a layer of Sertoli cells.
Theca cells. Cells in the outermost layer of the ovarian follicle that produce androgen as a source for neighbouring granulosa cells to convert to estrogens.
WT1(+KTS)/WT1(–KTS). Isoforms of the Wilms tumour suppressor protein (WT1) that have three amino acids (lysine, threonine, serine: KTS) inserted or excluded, respectively.
ZZ/ZW sex determining system. A system in which males have two identical sex chromosomes and females have two different sex chromosomes, in contrast to the situation with an XXXY sex-determining system.

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mammalian sex determination has come from studies in mice. In this primer, we summarize the results of these studies and discuss the insights they have provided into the molecular and cellular biology of mammalian testis development.

**An overview of the mammalian sex determination pathway**

The most detailed studies of mammalian sexual determination have been carried out using mice as a model (Fig. 1). In mice, the gonadal primordia, which are called the genital ridges (see Glossary, Box 1) arise at 10.0 days post coitum (dpc). At this stage, there are no morphological or functional differences between male and female genital ridges, and both structures contain precursor cells that have the ability to differentiate into Sertoli cells (which support germ cells in the testis, see Glossary, Box 1) or granulosa cells (which have a cognate role in the ovary, see Glossary, Box 1).

Sex-specific gonadal development is triggered by Sry expression in somatic gonadal cells of XY genital ridges at 10.0-10.5 dpc (Fig. 2A). SRY activity upregulates Sox9 (SRY box containing gene 9) transcription in Sertoli cell precursors, which in turn upregulates other genes involved in the differentiation of Sertoli cells. Logically, SRY activity must also directly or indirectly suppress the female sex-determining pathway, which would otherwise continue to be active, as it is in XX genital ridges. Differentiating Sertoli cells then assemble into testis cords (tubular structures that contain the germ cells; see Glossary, Box 1) (Fig. 2). The Sertoli cells then stimulate the sex-specific development of germ cells, androgen-producing Leydig cells (see Glossary, Box 1), testis vascular cells and other interstitial (i.e. non-cord) cell types. The formation of testes is the hallmark of male sex determination.

By contrast, in the absence of Sry in XX gonads, genes such as Wnt4 (wingless-type MMTV integration site family, member 4) and Foxl2 (forkhead box L2) start to be expressed in a female-specific manner and upregulate other downstream female genes, such as follistatin. The female-specific programme of gene expression leads to the differentiation of granulosa cells and theca cells (see Glossary, Box 1), the production of oocytes, and the formation of ovarian follicles (Fig. 1). These events also occur in XY gonads that lack SRY function, supporting the key role for SRY in both activating the testis-determining pathway and suppressing the ovarian-determining pathway.

**SRY structure and function**

SRY is the founding member of the SOX (SRY-related HMG box) family of transcription factors. The SOX family is found throughout the animal kingdom, and comprises 20 members in mice and humans. SOX proteins have diverse roles in embryogenesis and in the development of many organs, typically acting as cell differentiation switches (reviewed by Bowles et al., 2000). SRY, like other SOX transcription factors, is characterized by a high mobility group (HMG; see Glossary, Box 1) DNA-binding domain (Fig. 3A). This domain binds to the sequence (A/T)CAAA(T/A) in the minor groove of DNA, inducing a 60-85° bend (Harley and Goodfellow, 1994) (Fig. 3B). Biochemical analysis of mutant human SRY protein from XY females has revealed that both DNA-binding and
-bending are essential for SRY function. Furthermore, most human XY females have mutations in the HMG domain, reflecting the importance of this domain for the function of SRY (reviewed by Harley and Goodfellow, 1994).

In contrast to the HMG domain, the remaining parts of SRY are poorly conserved between species, and no additional conserved functional domains have been identified (Fig. 3A). For example, in mice, only two amino acids make up the N-terminal region that usually comprises 30-60 amino acids in other species (Gubbay et al., 1990). In the C-terminal region of mouse SRY, a long glutamine (Q)-rich domain is present that is not found in other mammalian species but that might act as a transcriptional activation domain (Dubin and Ostrer, 1994). Sry transgene constructs that lack this domain fail to cause male development (Bowles et al., 1999), although it is not known whether this failure reflects a reduced stability of the truncated protein. XX transgenic mice that express human or goat SRY develop as males (Lovell-Badge et al., 2002; Pannetier et al., 2006), suggesting that the non-HMG-domain regions of SRY either are conserved in function but not in sequence, or have no function. In support of the former possibility, the human SRY protein has to be of full length to show normal DNA-binding ability in vitro (Sanchez-Moreno et al., 2008).

SRY protein also carries two nuclear localization signals (NLSs; see Glossary, Box 1) and target sites for acetylation and phosphorylation (Fig. 3A). The NLSs lie at each end of the HMG domain and are conserved between moue and human (Sudbeck and Scherer, 1997); these bind calmodulin (a calcium-binding protein) and importin β (a nuclear import protein), respectively (Harley et al., 1996; Forwood et al., 2001; Sim et al., 2005) (Fig. 3A). Mutations in either NLS can cause human XY sex reversal, indicating that they are not functionally redundant (Battiloro et al., 1997; Veitia et al., 1997; Harley et al., 2003; Sim et al., 2005). Human SRY is acetylated at a single lysine residue that is well-conserved between species (Fig. 3A); acetylation enhances the nuclear localization of SRY by facilitating its interaction with importin β (Thevenet et al., 2004). Furthermore, human SRY is phosphorylated by cAMP-dependent protein kinase (PKA) on serine residues (S31-S33) located in the N-terminal part of the protein. This PKA-dependent phosphorylation of SRY increases its DNA-binding ability and its subsequent transcriptional activity, and is conserved across primates (Desclozeaux et al., 1998). Other possible phosphorylation residues (serine or threonine) are conserved in the N-terminal domain of SRY in all eutherian mammals (see Glossary, Box 1), except rodents, but the precise function of these residues has not been elucidated.

A number of proteins, including KRABO (kruppel-associated box domain only) (Oh et al., 2005; Peng et al., 2009), WT1 (Wilms tumour 1) (Matsuzawa-Watanabe et al., 2003), SIP1 (SRY-interacting protein 1) (Poulat et al., 1997) and PARP1 [Poly (ADP-ribose) polymerase] (Li et al., 2006) have been shown to interact with mouse and human SRY (Fig. 3A). However, most studies of these protein-protein interactions to date have been based on in vitro systems, and so their physiological significance is as yet unclear.

In summary, our understanding of the molecular mode of action of SRY remains rudimentary. Available evidence points to it having a role as a transcription factor that enters the nucleus, binds to DNA and then upregulates the expression of Sox9 (see below), but detailed structure-function relationships and how SRY functions in different mammalian species, despite its high sequence divergence, remain to be determined.

**Sry expression and its regulation**

Most data on the regulation of Sry have been obtained from studies in mice, a species in which the expression of Sry in gonads is tightly regulated in both space and time. In the mouse embryo, Sry expression starts at 10.5 dpc in somatic cells of XY genital ridges, reaches a peak at 11.5 dpc and wanes by 12.5 dpc (Koopman et al., 1990; Hacker et al., 1995; Jeske et al., 1995; Bullejos and Koopman, 2001; Wilhelm et al., 2005) (Fig. 1, Fig. 2A). Its expression is associated with the differentiation of Sertoli cells in the testis but, clearly, continued Sry expression in mice is not needed to maintain the Sertoli cell phenotype.

In all other species studied, Sry expression in the gonads is maintained rather than transient, for reasons that are not clear. Furthermore, in some species, such as humans and wallabies, Sry expression is not limited to the gonads, and is instead expressed in many tissues during foetal development (Clepet et al., 1993; Harry et al., 1995). In these species, the function of SRY beyond its role in testis development has not been determined.
One of the unique characteristics of mouse Sry expression is its peculiar spatio-temporal wave-like pattern: Sry expression is initiated in the centre of the genital ridge before extending to the whole length of the gonad over a period of several hours (Fig. 2A) (Bullejos and Koopman, 2001; Wilhelm et al., 2005). Thus, not all parts of the genital ridge are exposed to Sry transcripts, or protein, at the same time. The transient and dynamic expression of Sry in mice has highlighted the concept that Sry functions within a critical window of time in individual somatic cells of the developing gonad. This concept was suggested after observations of the phenomenon known as B6-YDOM sex reversal, which arises when specific variants of the Y chromosome from the mouse species Mus domesticus (YDOM) are crossed onto the genetic background of the C57BL/6J (B6) inbred mouse strain. Repeated crossing to B6 mice leads to a variety of phenotypes, ranging from delayed testicular development to the development of ovotestes (see Glossary, Box 1) or ovaries (Eicher et al., 1982). Curiously, when ovotestes develop, they tend to have a testicular structure in the centre, flanked by ovarian regions (Eicher et al., 1982). Detailed in situ hybridization studies have demonstrated that a delay in initiating the expression of Sry underlies B6-YDOM sex reversal: the peak level of Sry expression in XY B6 Ypos gonads is delayed by up to 10 hours (Bullejos and Koopman, 2005). Thus, a critical window of time during which Sry can direct Sertoli cell development appears to close while the Sry expression wave is still confined to the central region of these XY gonads (Fig. 4A).

The existence of a narrow and crucial time window for the expression and function of Sry has been confirmed in transgenic mice that carry a Sry gene driven by the heat shock protein 70.3 (Hsp70.3) promoter, which allows for the experimental induction of Sry at various time points. In this system, a 6-hour delay of Sry induction results in a failure to initiate the testis development pathway (Hiramatsu et al., 2009). The limits of this window may be dictated by the need to pre-empt the pathway of ovarian granulosa cell development that occurs in the absence of Sry, or by the availability of partner proteins required for SRY protein to activate Sox9.

In contrast to the timing of Sry expression, the initiation of which is particularly important, the duration of Sry expression appears to be immaterial, as it varies between species. For example, in humans and goats, Sry gene expression persists well beyond sex determination and is observed even after birth (Hanley et al., 2000; Pannetier et al., 2006). Taken together, the crucial factor that determines the ability of SRY to induce Sertoli cell differentiation appears to be whether or not it is able to exceed a required threshold level of expression in any precursor cell within a given window of opportunity (Fig. 4A).

In addition to the threshold of Sry expression being required for Sertoli cell differentiation, there is another threshold to consider: the number of Sertoli cells required for proper testicular development. In experiments in which chimaeras were generated by combining XX and XY early mouse embryos, gonads containing various proportions of XY cells were produced. When fewer than 10% were XY, ovaries were formed, but when 35-40% of somatic cells were XY, testes were formed, but when a few percent of both were XY, ovaries were formed (Burgoine et al., 1988) (Fig. 4B). Intermediate proportions of XY cells resulted in ovotestes.

A further observation to arise from these studies was that most, but not all, Sertoli cells in XX→XY chimaeras were XY (Palmer and Burgoine, 1991). These findings suggested that SRY expression normally drives Sertoli cell differentiation; however, it was also clear that SRY expression might not be an absolute requirement, and that XX cells could be recruited to the Sertoli cell population, perhaps by paracrine signalling (see Glossary, Box 1). More recently, studies of normal mouse testicular development using Sox9 and SRY antibodies have revealed the existence of pre-Sertoli cells (see Glossary, Box 1) that express Sox9 without
having first expressed SRY (Wilhelm et al., 2005). Moreover, in in vitro cultures that contain a mix of XX genital ridge cells that constitutively express a fluorescent marker and XY wild-type genital ridge cells, it has been shown that when the XY cells are in contact with the XX cells, the XX cells are induced to express Sox9, confirming the existence of a paracrine recruitment mechanism (Wilhelm et al., 2005). This phenomenon can be artificially induced by prostaglandin D2 (PGD2), and inhibited by a chemical blocker of the PGD2 receptor, implicating PGD2 in this paracrine recruitment mechanism (Wilhelm et al., 2005). Accordingly, Pgd2 (prostaglandin D synthase)-knockout mice show decreased Sox9 expression in male gonads (Moniot et al., 2009). This mechanism is likely to provide a backup system to ensure and to reinforce male pathway activation by SRY.

In spite of the importance of its correct temporal and spatial expression, the regulation of Sry is still poorly understood. Regions that flank Sry are remarkably poorly conserved between mammalian species, hampering efforts to find potentially important regulatory elements. Although sequence analysis has revealed four intervals of relatively high DNA sequence conservation upstream of Sry among human, bovine, pig and goat genomes (Ross et al., 2008), the physiological significance of these sequences has not been determined. Transgenic mouse assays (in which critical regulatory regions are pinpointed by sequential deletion analysis) have so far not been useful for identifying a gonadal enhancer of Sry.

Despite these complications, several proteins have been implicated in regulating Sry expression (Table 1). Analyses of gene knockout mouse models have shown that the absence of these proteins leads to reduced levels of Sry expression, and results in XY sex reversal. In most cases, it is not clear whether the loss of these factors reduces the level of Sry expressed per cell or the overall number of cells that express Sry. How loss of their function leads to reduced Sry is also unclear for most of these proteins. For example, Fog2 (friend of GATA2; now known as ZFPM1 – Mouse Genome Informatics) is known to be involved in the repression rather than in the activation of several GATA-dependent target genes, such as anti-Müllerian hormone (Amh) and inhibin α (Robert et al., 2002). CBX2 (chromobox homologue 2), MAP3K4 (mitogen-activated protein kinase kinase kinase 4) and insulin receptors are not transcription factors, and so how they might influence Sry regulation remains to be determined. A recent bioinformatics study identified a region of mouse chromosome 1 between 33 and 49 cM that controls the expression of Sry, but none of the above genes is located within this region (Munger et al., 2009).

Among the molecules described above, much attention has focused on WT1 as a potential regulator of Sry. When WT1(+KTS) (see Glossary, Box 1) is reduced in humans, XY sex reversal occurs, accompanying a condition known as Frasier syndrome (Barbaux et al., 1997). Studies in mice show that

Table 1. Genes implicated in regulating Sry expression

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<thead>
<tr>
<th>Gene</th>
<th>Type of protein</th>
<th>Loss-of-function phenotype</th>
<th>References</th>
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<tr>
<td>Wt1 (+KTS)</td>
<td>Transcription factor/RNA-binding protein</td>
<td>Frasier syndrome (XY sex reversal, glomerulonephropathy)</td>
<td>Barbaux et al., 1997</td>
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<td>XY sex reversal, dysplastic kidney</td>
<td>Hammes et al., 2001</td>
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<td>Cbx2</td>
<td>Transcriptional co-factor</td>
<td>XY sex reversal</td>
<td>Katoh-Fukui et al., 1998</td>
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<tr>
<td>Gata4/Fog2</td>
<td>Transcription factor/co-factor (co-repressor)</td>
<td>XY sex reversal, skeletal defects</td>
<td>Biason-Lauber et al., 2009</td>
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<tr>
<td>Map3k4</td>
<td>Kinase (enzyme)</td>
<td>*</td>
<td>Tevosian et al., 2002</td>
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<tr>
<td>Ir, Irr, Igf1r†</td>
<td>Membrane receptors</td>
<td>*</td>
<td>Bogani et al., 2009</td>
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<td>XY sex reversal, foetal lethal</td>
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<td>XY sex reversal, spina bifida</td>
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<td>XY sex reversal, neonatal death</td>
<td>Nef et al., 2003</td>
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†No mutations in human sexual reversal patients identified.
†Loss-of-function phenotype observed in triple knockout mice.
Cbx2, chromobox homolog 2 (also known as m335); Fog2, friend of GATA2; Gata4; Gata binding protein 4; Igf1r, insulin-like growth factor 1 receptor; Ir, insulin receptor; Sry, insulin receptor-related receptor; Map3k4, mitogen-activated protein kinase kinase kinase 4; WT1(+KTS), Wilms tumour 1 gene, encoding an isoform that includes a three-amino acid motif (lysine-threonine-serine).
WT1(+KTS) plays a cell-autonomous (see Glossary, Box 1) role in regulating Sry expression in individual cells of the Sertoli cell lineage in vivo (Bradford et al., 2009b), but it is not clear whether Sry is a direct or indirect transcriptional target of WT1. In in vitro systems, WT1(+KTS) activates the promoter of mouse but not of human Sry (Shimamura et al., 1997; Hossain and Saunders, 2001; Miyamoto et al., 2008); moreover, WT1(+KTS) preferentially binds to unspliced pre-mRNAs, promoting gene expression by mRNA processing (Morrison et al., 2008). Thus, the mechanism of Sry regulation by WT1(+KTS) is unclear and requires further study.

In summary, the precise regulation of both the levels and onset of Sry expression is important for Sertoli cell differentiation and hence for testis development. The mechanistic details of how this regulation is achieved, however, remain unknown.

**Mechanism of SRY action**

After the discovery of Sry, attention turned to the issue of whether it might directly upregulate the male sex-determining pathway, or repress a repressor of that pathway (see Box 2). Much of this work focused on the relationship between Sry and Sox9, another important and early-acting gene that is expressed in Sertoli cells. In particular, studies have aimed to address whether SRY might regulate Sox9 expression directly or indirectly, and whether Sox9 might be just one of many targets of SRY.

**Sox9: a major SRY target**

Sox9, which encodes another member of the SOX transcription factor family, is expressed in several developing vertebrate organ systems, including the skeleton, heart, kidneys and brain (Wright et al., 1995). In XY mouse gonads, Sox9 expression is upregulated in pre-Sertoli cells immediately after the onset of Sry gene expression, and mimics the wave-like pattern of Sry expression (Kent et al., 1996; Morais da Silva et al., 1996; Sekido et al., 2004; Bullejos and Koopman, 2005; Wilhelm et al., 2005). However, unlike Sry in mice, Sox9 expression persists in the gonad beyond 12.5 dpc (Fig. 2B) (Kent et al., 1996; Morais da Silva et al., 1996), suggesting that it is able to sustain its own expression once initiated (Fig. 1), and that its continued expression might be linked to the maintenance of the Sertoli cell phenotype (DInapoli and Capel, 2008).

The importance of Sox9 for sex determination was revealed when heterozygous human Sox9 mutations were found to be associated with a skeletal deformity syndrome: campomelic dysplasia (CD) (Foster et al., 1994; Wagner et al., 1994). In 75% of XY individuals with CD, partial or complete male-to-female sex reversal occurs, indicating that Sox9 expression is important for testis determination in humans (Mansour et al., 1995). In addition, a duplication of 11-13 kb upstream of the Sox9 gene does not have a ZZ/ZW sex-determining system (see Glossary, Box 1) and mimics the wave-like pattern of Sox9 expression in individual cells of the Sertoli cell lineages (Spotila et al., 1998; Western et al., 1999). Therefore, the role of Sox9 in sex determination is considered to be ancestral and pivotal among vertebrates. Furthermore, because the phenotype of Sox9 overexpression in mouse XX gonads recapitulates that of Sry overexpression, Sox9 is thought to be the only gene that is required downstream of Sry to activate the remainder of the testis-determining programme.

How does SRY upregulate Sox9 expression? A recent analysis using transgenic reporter assays and in vivo chromatin immunoprecipitation (ChiP, see Glossary, Box 1) assays identified a gonad-specific enhancer of mouse Sox9, called TESCO (for testis-specific enhancer of Sox9 core) (Sekido and Lovell-Badge, 2008). TESCO is a 1.4 kb sequence that is located 11-13 kb upstream of the Sox9 transcription start site and is highly conserved in rodent, dog, and human genomes. Sox9 and SF1 (steroidogenic factor 1), an orphan nuclear receptor that regulates many genes involved in the differentiation of gonadal and adrenal cells, were found to cooperatively upregulate mouse Sox9 by directly binding to TESCO (Sekido and Lovell-Badge, 2008) (Fig. 5), providing the first evidence that SRY acts as a transcriptional activator in vivo.

The discovery of TESCO also clarified another significant mechanism of Sox9 regulation: an auto-regulatory positive-feedback loop (Fig. 5). Sox9 itself binds to TESCO with SF1 to stimulate its continued expression (Sekido and Lovell-Badge, 2008). This positive feedback loop is important not only to maintain Sox9 expression after Sry subsides in mice, but also to ensure that the SRY signal is captured and amplified in each developing Sertoli cell. A positive-feedback loop is also observed between fibroblast growth factor 9 (FGF9) and Sox9 (Kim et al., 2006). Fgf9 has not been proven to be a target of Sox9, but is known to be necessary for the maintenance of Sox9 expression.

**Box 2. The quest to find the molecular function of SRY**

Despite being long suspected, it took over a decade to prove that SRY (sex-determining region on the chromosome Y) regulates Sox9 (SRY box containing gene 9) directly. Why did it take so long? Sry has some peculiar features that make it particularly difficult to work with. The non-HMG domain sequence of mouse Sry gene does not have obvious functional domains and is poorly conserved between species. As a result, it was not known whether SRY acts as a transcriptional activator or a repressor, or even as an architectural factor that acts by changing DNA structure. The fact that SRY binds to a short (seven-base) target sequence that occurs frequently in the genome made it difficult to identify potential target genes bioinformatically. Furthermore, a lack of suitable cell lines and antibodies hampered molecular approaches. Useful and specific antibodies to SRY now exist, although cell lines remain a problem, as cells typically lose SRY expression after a few passages in culture.

Nor has it been easy to work backwards from Sox9 to find out what regulates its expression, or whether SRY is involved. Translocations and deletions affecting Sox9 function indicate that regulatory elements exist 1 Mb to 10 Mb upstream of this gene in mice and humans (Bishop et al., 2000; Pfeifer et al., 1999). Painstaking deletion analysis in transgenic Sox9 reporter mice finally revealed the gonadal-specific enhancer of Sox9, called testis-specific enhancer of Sox9 core (TESCO), on which SRY acts directly (Sekido and Lovell-Badge, 2008). Even now it is not clear whether TESCO is the only element important for Sox9 regulation during sex determination.
**Box 3. Sry and sex-specific brain function**

In mice, Sry (sex-determining region on the chromosome Y) is not only expressed in developing gonads, but also in the brain. It is tempting to hypothesise that SRY has some direct role in brain sex determination. So far, a few genes encoding proteins such as monoamine oxidase A (MAO-A) and tyrosine hydroxylase (TH), have been identified as potential targets of SRY in the brain (Milsted et al., 2004; Dewing et al., 2006; Wu et al., 2009). However, the physiological significance of these pathways and the regulatory mechanisms of Sry in the brain have yet to be elucidated. Because so little is known about sex-specific brain differentiation, and because the topic itself is fraught with controversy, it is a challenging task to prove that SRY has a direct role.

**Additional SRY targets in gonads**

Other than Sox9, few SRY target molecules in gonads have been reported. A recent in vivo ChIP study showed that the cerebellin 4 precursor gene (Chln4) is a direct target of mouse SRY (Bradford et al., 2009a). Chln4 encodes a transmembrane protein and is expressed in a male-specific manner. However, the function of Chln4 product in testicular development is not known.

Human and mouse SRY have also been found to interact with β-catenin and to repress β-catenin-mediated TCF-dependent gene activation (Bernard et al., 2008; Tamashiro et al., 2008; Lau and Li, 2009). β-Catenin is the downstream effector molecule of WNT4/R-sponsin 1 (RSPO1) signalling and appears to be essential for initiating the development of the female gonad (Maatouk et al., 2008). It is attractive to speculate that, in order to activate the male pathway efficiently, SRY suppresses the function of the key female molecule β-catenin. However, evidence that SRY affects β-catenin function is based solely on in vitro data and its physiological significance in vivo has not yet been demonstrated.

In summary, the only clear direct target of SRY during testis determination is Sox9, and so understanding the role of SRY in engaging the testis-determining pathway becomes an issue of studying the molecular roles of Sox9. Interestingly, in mice, Sry is also expressed in the brain, where other potential targets of its protein have been identified (see Box 3); these targets are brain specific and so are not relevant to gonadal development.

**Conclusion**

Despite its dramatic biological role, Sry is a fragile and partly debilitated gene. The structure and regulatory sequences of Sry may have been degraded because of its location on the rapidly degrading Y chromosome (see Box 4). To respond to the weak Sry expression signal and to establish the male pathway efficiently, Sox9, apparently the only meaningful target of SRY, has acquired support mechanisms for its own regulation, in the form of cell-autonomous and intercellular signalling-based positive-feedback loops. Thus, SRY provides the trigger for male sex determination.
Box 4. Y-chromosome evolution and Sry
Sry (sex-determining region on the chromosome Y) determines the sexual fate of the organism, but also the fate of the Y chromosome itself. Gene-mapping analysis suggests that the X and Y chromosomes evolved from a pair of identical chromosomes (Graves, 2006) that began to differentiate when one copy of Sox3 (SRY box containing gene 3) acquired a new function in male sex determination. The chromosome carrying this new gene, Sry, became the Y chromosome. The ancestral Sox3, the role of which is largely restricted to brain development, resides on what has become the X chromosome.

Having established a new Y chromosome, subsequent selection favoured restricted recombination with the new X, so that Y genes would stay on the Y chromosome. Because recombination is part of the mechanism used by cells to proofread and repair genes, genes in the non-recombining region of the Y chromosome tend to become lost and/or degraded. As a result, the human Y chromosome has shrunk to ~60 Mb and contains only 50 functional genes, whereas the human X chromosome is ~165 Mb and carries ~1000 genes (Graves, 2002; Graves, 2006; Wallis et al., 2008). Erosion of the Y chromosome is reflected in the accumulation of Sry mutations, explaining the high level of sequence divergence between mammalian species and perhaps accounting for its relatively low expression levels in mice. Not surprisingly, the use of Sry as a testis-determining trigger seems to have been lost altogether in some species, such as the mole vole E. lutescens and the Japanese spinous rat T. osimensis, which do not develop testis-determining program. At the same time, SOX9 provides a mechanism by which it does actually SOX9, and not SRY, that is the key element that orchestrates and stabilizes Sertoli cell differentiation to lock in the testis-determining program. At the same time, SOX9 provides a means of blocking the pathway of gene activity that leads to the differentiation of ovarian cells, but the mechanism by which it does so remains unclear.

Intensive studies spanning more than 20 years since the discovery of Sry have identified several genes important for sex determination and gonadal development, and yet 30-40% of human XY DSD cases still remain undiagnosed. Given the importance of cavitation of the MEIS and DSDs, further study is required to test this possibility. Recent technical advances, including bioinformatic analysis and the generation of genetically modified mice, will help to answer the remaining questions surrounding the process of mammalian sex determination, and may provide valuable information for the diagnosis and management of human DSDs.

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