REVIEW ARTICLE

Understanding sex determination in the mouse: genetics, epigenetics and the story of mutual antagonisms

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Abstract

Recent years have seen a rapid growth in mouse genetics resources that support research into fundamental mechanisms in organogenesis, including those controlling mammalian sex determinations. Numerous mouse mutants have shed light on molecular pathways of cell fate specification during gonadogenesis and the 'decision' as to whether testis or ovary development is achieved. These studies indicate substantial genetic complexity, characterized by redundancy, feedback loops, mutual antagonism between testis-determining and ovary-determining gene regulatory networks and a degree of plasticity in the fully differentiated state of the adult gonad that was not appreciated until conditional loss-of-function studies were performed. One challenge now is to understand how controlled epigenomic changes effect the now familiar sexually dimorphic transcriptomic profiles of the male and female gonads, firstly during primary sex determination, but also in the adult gonad, thereby regulating cellular behaviour during morphogenesis and maintaining the differentiated state.

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'When Mary and I were discussing the option of my coming to Harwell in 1969, I mentioned that she might not believe me—and may think twice about my joining the Unit—when I said I was working on an inherited condition giving rise to XX males (here, I was referring to the start of my *Sxr* work). She wrote back saying that, yes, she would believe it, if I could believe that she was working on an inherited condition giving rise to XY females. This, of course, was the *Tfm* female. I have always thought this an amusing example of Mary's sense of humour.' Dr Bruce Cattanach, personal communication, April 2015.

Introduction

In this overview of the genetics of mammalian sex determination, I will highlight three aspects of our current understanding that we owe most obviously to the analysis of mouse mutants. In the first section, I will consider the mutual antagonisms that exist between the testis-determining and ovary-determining gene regulatory networks during embryonic sex determination and how these might be mediated. Next, I will consider the role of genes that act to maintain the differentiated state of the adult gonads and their relationship to genes that function in primary sex determination. Finally, I will consider evidence for the role of chromatin modifiers in testis determination and the growing interest in the epigenomics of sex determination. By necessity, such a review cannot be completely comprehensive due to space constraints—I will focus on commitment of the supporting cell lineage and omit discussion of two other key lineages: germs cells and steroidogenic cells. But I hope that it will make clear how the central concerns of Mary Lyon's research at Harwell—using mouse genetics to shed light on mechanisms of gene regulation and their epigenetic control, are also important elements in contemporary mammalian sex determination studies.

Mutual distrust: antagonisms between genes required for testis and ovary development

The mouse gonad forms initially, at around 10.0 days *post coitum* (dpc), on the surface of the mesonephros as a bipotential primordium with the capacity to develop as a testis or ovary depending on the expression of specific genes during a key developmental window. The first gonadal lineage in which this commitment is evident is the somatic supporting

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cell lineage: pre-Sertoli cells develop in gonads committed to the testicular fate, while pregranulosa cells indicate a commitment to the ovary. Sertoli and germ cells are subsequently confined to the newly formed testis cords and the steroidogenic Leydig cells differentiate in the interstitial region. Testes form in XY embryos due to a pathway of gene expression that is initiated by the expression of the Y chromosome gene, *Sry*, in supporting cell precursors of the embryonic gonad (figure 1a). This process of embryonic gonadal fate commitment is known as primary sex determination. *Sry* transcripts are detected for a short period of time during gonadogenesis, from around 10.5 to 12.5 dpc (Bullejos and Koopman 2001). SRY, an HMG-box DNA-binding protein, which is required to act during this short window of opportunity to prevent ovary development (Hiramatsu *et al.* 2008), binds an enhancer of the *Sox9* gene and results in its upregulation (Sekido and Lovell-Badge 2008). SOX9, also an HMG-box transcription factor, is necessary and sufficient for testis determination and is central to the differentiation of Sertoli cells (Vidal *et al*. 2001; Barrionuevo *et al*. 2006). A variety of SOX9 target genes have been described, some of which presumably act to reinforce commitment of the supporting cell lineage to the Sertoli cell fate (Jakob and Lovell-Badge 2011). In the absence of the Y chromosome, such as in XX and XO embryos, the supporting cell lineage commits to the granulosa cell fate and ovary development ensues due to a combination of genetic pathways acting in concert (see below).

Sertoli cells act as organizing centres for the variety of morphogenetic processes required to build a functioning testis (Cool *et al.* 2012). Loss of either SRY or SOX9 function results in XY gonadal sex reversal; gain of function of SRY or SOX9 in transgenic XX gonads results in testis development (reviewed in Sekido and Lovell-Badge 2009; Kashimada and Koopman 2010; Jakob and Lovell-Badge 2011; Warr and Greenfield 2012). Analysis of mouse mutants revealed that, after its initial upregulation, maintenance of SOX9 expression at high levels in Sertoli cells requires fibroblast growth factor (FGF) signalling (Kim *et al.* 2006). FGF9 acts diffusibly to rapidly spread masculinizing signals, which are initiated in the centre of the bipotential gonad by *Sry* expression (Bullejos and Koopman 2001), to the gonadal poles in a paracrine fashion (Hiramatsu *et al.* 2010). This centre-to-pole temporal profile has an anatomic consequence: any delay in the receipt of these masculinising signals at the poles of the gonad can result in the characteristic pattern of ovotestis development, with polar ovarian tissue either side of a central testicular region (Bogani *et al.* 2009; Wilhelm *et al.* 2009). Other noncell-autonomous signals, such as prostaglandin D2 (Moniot *et al.* 2009), may act to recruit cells to the Sertoli cell fate by enhancing SOX9 expression. The timing of such signals is important: the newly formed gonad has an innate bias towards the expression of genes subsequently required for ovary development, whose activity must be inhibited for testis determination and differentiation to proceed normally (Jameson *et al.* 2012b).

While initially thought to act positively in regulating the expression of *SOX9*, genetic studies of a double knockout of *Wnt4* and *Fgf9* indicate that FGF9 acts, through its receptor FGFR2 (Bagheri-Fam *et al.* 2008; Colvin *et al.* 2001; Siggers *et al.* 2014), in a predominantly negative fashion—suppressing the activity of canonical WNT signalling (Jameson *et al*. 2012a). This antagonism of canonical WNT signals, which are themselves required for normal ovary development by opposing testis-determining signals (Vainio *et al.* 1999; Kim *et al.* 2006), is a common theme in mouse sex determination. Canonical WNT signals, in the form of RSPO1, WNT4 and β-catenin, antagonize SOX9 activity and vice versa (Kim *et al.* 2006; Chassot *et al.* 2008; Maatouk *et al.* 2008; Lavery *et al.* 2012). Again, the study of double knockouts has revealed how complex the interactions between these pathways are, and how functional redundancy can also operate to nullify or ameliorate the deleterious impact of gene deletion. Further research will be required to understand how these mutual antagonisms (summarized in figure 1a) identified genetically are controlled at the molecular level. Their evolution likely reflects the requirement to canalize development of a bipotential organ to just one of two possible fates.

Maintenance kit: the plasticity of the adult gonads

The focus on primary sex determination and the identification of molecules required for it perhaps led the field to think that, once embryonic gonadal sex was established, the so-called 'battle of the sexes' was over. Therefore, it came as a surprise when two ground-breaking papers described conditional loss-of-function studies in mice revealing that the same molecules implicated in primary sex determination and sexual differentiation in a variety of species were also required to maintain the differentiated state of the adult gonad.

Female maintenance: FOXL2

The forkhead transcription factor FOXL2 is one of the earliest known markers of ovary differentiation in the mouse, with expression detectable from 11.5 dpc (Bogani *et al.* 2009). However, it is not required for primary sex determination: mouse gonads lacking *Foxl2* are overtly normal until birth (Schmidt *et al.* 2004; Uda *et al.* 2004). This is in contrast to another mammal, the goat, in which gene deletion of *FOXL2* at the fertilized oocyte stage results in XX sex reversal associated with upregulation of SOX9 in the foetal gonad (Boulanger *et al.* 2014). However, further studies in postnatal mouse ovaries lacking FOXL2 revealed upregulation of *Sox9* expression, suggesting the possibility of an ongoing requirement for FOXL2 to maintain ovarian cell identity (Ottolenghi *et al.* 2005). Subsequently, it was shown that induced deletion of *Foxl2* in adult ovarian follicles caused

a reprogramming of granulosa and theca cells into Sertoli-like and Leydig-like cells (Uhlenhaut *et al.* 2009). Evidence suggests that FOXL2 can inhibit *Sox9* expression, in cooperation with oestrogen receptors, by acting through the TESCO gonadal enhancer (Uhlenhaut *et al.* 2009). These data indicate that continued antagonism of protestis genes is a requirement for maintenance of the differentiated state of the adult ovary (see summary in figure 1b).

Male maintenance: DMRT1

Proteins containing a DM domain DNA-binding motif have been implicated in sex determination in a number of invertebrate species (reviewed in Matson and Zarkower 2012). The sex-determining genes *doublesex* and *mab-3* from *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, were the first members of this gene family to be identified. The human homologue, *DMRT1*, is found on a chromosomal region (9p24.3) for which hemizygosity is associated with disorders of sex development (DSD), including XY female individuals. It was therefore somewhat surprising that XY mice lacking *Dmrt1* develop testes as normal and are born as male (Raymond *et al.* 2000). It was subsequently shown that DMRT1 functions in complex ways in a number of male and female gonadal cell-types to support sexual differentiation.

From around two weeks after birth, *Dmrt1*-deficient XY testes begin to lose the Sertoli cell marker, SOX9, and acquire FOXL2 instead, revealing evidence of transdifferentiation of Sertoli cells to granulosa cells (Matson *et al.* 2011). A direct test of DMRT1's role in maintenance of the differentiated testicular state was performed by deletion of *Dmrt1* in adult mouse Sertoli cells (Matson *et al.* 2011). The result was a partial reprogramming of this testcular cell-type to a granulosa-like cell fate and the appearance of theca-like cells in mutant XY gonads. The adult XY gonad requires DMRT1 to inhibit, directly or indirectly, the expression of ovary-determining genes, some of which, but not all, are associated with embryonic ovary development (figure 1b) (Matson *et al.* 2011; Minkina *et al.* 2014). It is also interesting to note that forced expression of DMRT1 in XX ovarian cells *in vivo* using distinct methods can either induce postnatal reprogramming of these to Sertoli-like cells (Lindeman *et al.* 2015) or, quite surprisingly given the absence of a clear role in primary sex determination, drive testicular differentiation at the foetal stage (Zhao *et al.* 2015). It is sometimes difficult to interpret such gain-of-function experiments, but they indicate that the mouse gonad retains a capacity, in common with many other vertebrate species, to respond to DMRT1 during primary sex determination if it is expressed at the right time, in the right place and at the appropriate levels.

In summary, there exists an ongoing reciprocal antagonism between male-promoting and female-promoting gene/protein networks that is crucial for maintenance of the differentiated gonadal state in adults (figure 1b). There are genes in common between the networks that operate during embryonic sex determination and maintenance of adult gonadal cell fate, suggesting that the function of several sex-determining genes is an ongoing requirement. Full details of the similarities and differences between determination and maintenance networks remain to be elucidated.

Modelling agencies: chromatin modifying proteins and testis determination

Primary sex determination and its postnatal reinforcement, and stabilization are associated with sexually dimorphic programmes of transcription. Underlying these divergent programmes there are likely to exist epigenomic modulations that are presumably as diverse and dynamic as the gene expression profiles they underpin. Careful, genomewide characterization of the many epigenomic events—including methylation of cytosine residues of CpG dinucleotides and histone posttranslational modifications—that occur during the transition of the bipotential supporting cell precursor to either the Sertoli or granulosa cell fate is hampered by the limiting number of embryonic gonadal cells available for biochemical studies. However, loss-of-function studies in the mouse suggest key roles for chromatin modellers in testis determination, with a focus on the regulation of *Sry* expression.

The GADD45 protein family has been implicated in locus-specific, active DNA demethylation in a number of systems (Niehrs and Schafer 2012). XY mice lacking GADD45γ on the C57BL/6 background undergo complete gonadal sex reversal associated with delayed *Sry* expression (Gierl *et al.* 2012; Warr *et al.* 2012; Johnen *et al.* 2013). This led to the hypothesis that hypomethylation of a small number of CpG dinucleotides in the *Sry* promoter region, which is restricted to gonadal somatic cells and therefore indicative of *Sry* activity (Nishino *et al.* 2004), might be disrupted in mutant XY gonads. This proved not to be the case (Gierl *et al.* 2012; Warr *et al.* 2012). Instead, the positive role played by GADD45γ in activating mitogen-activated protein kinase signalling, through MAP3K4 and p38 MAPK, was associated with enhanced phosphorylation of GATA4, a known transcriptional regulator of *Sry* (Gierl *et al.* 2012; Warr *et al.* 2012). However, the possibility that GADD45γ acts to control methylation of other regions of the *Sry* locus, or other loci entirely, was not ruled out. Recent studies describing MAPK recruitment to chromatin (Klein *et al.* 2013) suggest the possibility that epigenomic events controlled by a GADD45γ/MAP3K4/p38 MAPK signalling pathway act to control the timing of *Sry* expression. Further research is required to identify all targets of this signalling and the molecular mechanisms by which timing is so exquisitely controlled.

Further evidence of the importance of chromatin remodelling in *Sry* expression came from the observation that XY mice lacking the histone demethylase, JMJD1A, could

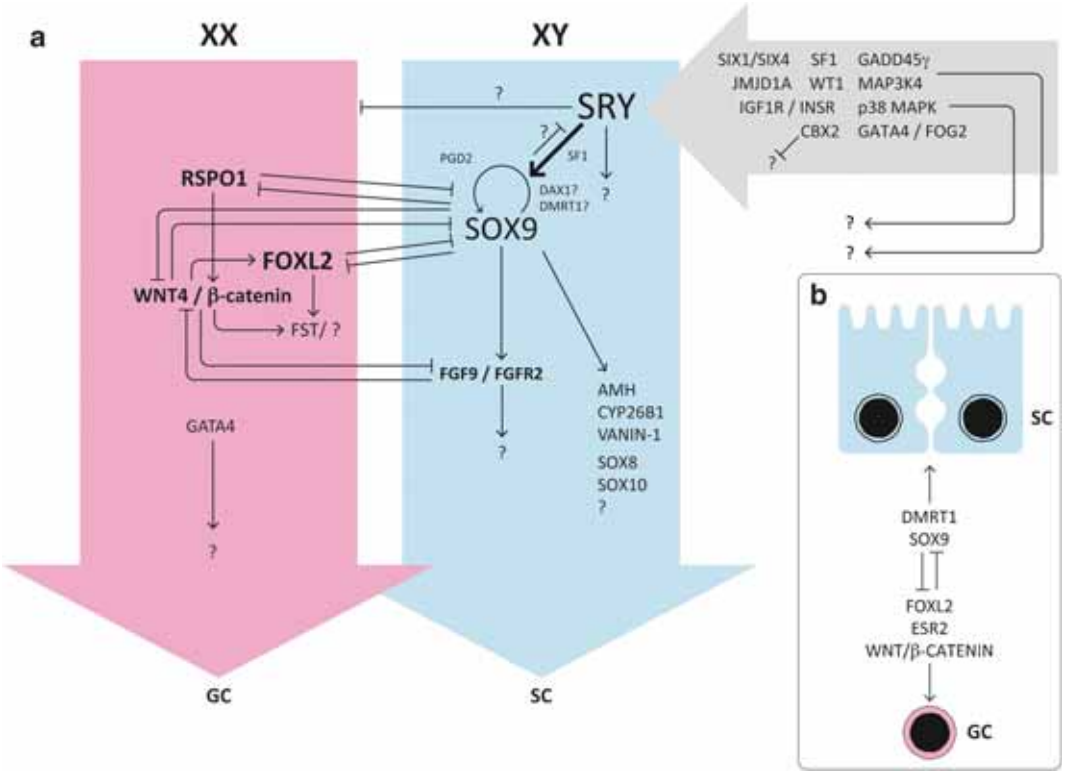

Figure 1. Ovary-determining and testis-determining gene regulatory networks that control primary sex determination, and maintain the identity of differentiated adult gonads, are mutually antagonistic. (a) Primary sex determination is dependent on the presence or absence of the Y-linked gene, *Sry,* in XY embryonic gonads. SRY in supporting cells upregulates *Sox9* expression, resulting in the development of Sertoli cells (SC). SOX9 acts by positively regulating (arrows) the expression of downstream targets such as *Amh*, as well as by inhibiting (hammered lines) the activity of ovary-determining genes and gene products. These, in contrast, encode proteins that inhibit testis-determining factors and promote ovary differentiation. In XX gonads, which lack *Sry*, canonical WNT signalling and FOXL2 promote the differentiation of granulosa cells (GC). A number of molecules are known to be required for normal *Sry* expression and thus necessary for testis determination (silver arrow). Question marks indicate hypothetical regulatory functions. (b) In adult ovarian somatic cells, FOXL2, in conjunction with oestrogen receptor and WNT signals, antagonizes the activity of *Sox9* and *Dmrt1* to maintain cellular identity. Loss of FOXL2 in such cells results in granulosa cell-to-Sertoli cell reprogramming. In contrast, DMRT1 and SOX9 act to inhibit pro-ovary genes to maintain Sertoli cell identity.

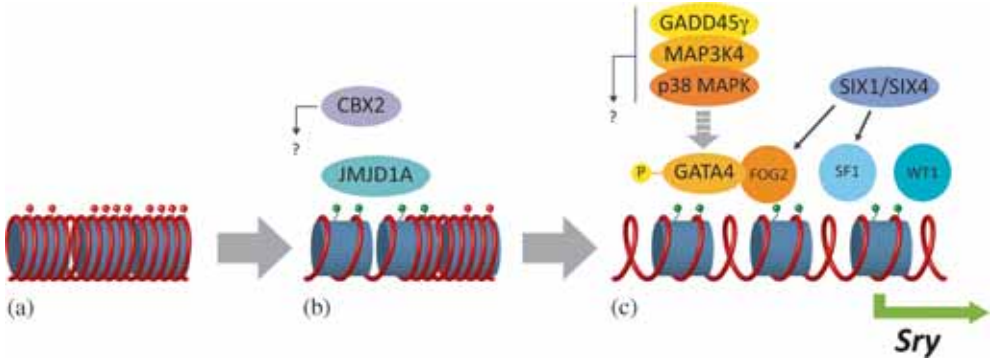

Figure 2. A model of the molecular control of *Sry* expression. (a) Histone H3K9 methylation (red circles) of *Sry* chromatin in regulatory regions is associated with transcriptional silencing (or heterochromatinization). (b) The H3K9 demethylase JMJD1A is recruited to *Sry* and demethylates H3K9 to promote an open chromatin configuration (or deheterochromatinization). Such an open configuration is also associated with higher levels of H3K4 methylation (H3K4me2, green circles). CBX2 also acts to promote *Sry* expression but the mechanistic basis of this is unclear. (c) Timely expression of *Sry* requires the activity of a GADD45γ-MAP3K4-p38 MAPK signalling module associated with enhanced phosphorylation of the transcription factor GATA4. Other targets of this signalling module may exist. Several other transcription factors are shown that impact directly or indirectly on *Sry* expression. Cis-regulatory sequences required for control of the spatiotemporal profile of *Sry* remain to be functionally specified.

exhibit gonadal sex reversal on certain genetic backgrounds (Kuroki *et al.* 2013). This sex reversal is associated with greatly reduced *Sry* expression at 11.5 dpc. JMJD1A acts specifically to demethylate histone H3K9, a negative chromatin mark associated with heterochromatin. Chromatin immunoprecipitation (ChIP) studies revealed that JMJD1A is recruited to *Sry* in gonadal somatic cells and that H3K9 methylation levels are elevated at *Sry* in its absence; in contrast, levels of H3K4 methylation, a positive mark, were decreased in mutant gonadal cells. These data support a model whereby JMJD1A-mediated H3K9 demethylation, a form of deheterochromatinization, is a prerequisite for subsequent methylation of H3K4 and the recruitment of transcription factors to permit *Sry* transcription (see figure 2). However, the regulation of *Sry* expression is a complex, evolving story (Larney *et al.* 2014). CBX2, a member of the polycomb group of proteins that exert their effects by modulating chromatin states, is also required for normal *Sry* expression and testis determination (Katoh-Fukui *et al.* 2012). The mechanism by which CBX2 controls *Sry* is unclear. Numerous other transcription factors have been implicated in *Sry* control (figure 2), and it will be important to discern which of these act directly on the *Sry* locus, versus indirectly—either by controlling expression of additional upstream factors or, alternatively, by regulating the number of SRY-positive supporting cell precursors in the XY gonad (reviewed in Warr and Greenfield 2012).

Overview and future directions

Mouse genetics is providing increasingly sophisticated tools for the study of sex determination and gonad development. Genes can be activated or inactivated in a cell-type-specific and stage-specific fashion to address the role they play in particular processes. Genome editing tools, such as CRISPR/Cas9, are also accelerating the delivery of subtle mutations of the mouse genome, and even the ability to modulate the epigenome in a controlled fashion (Hilton *et al*. 2015). Next generation sequencing permits genomewide surveillance of transcriptomic and epigenomic dynamics. It seems likely that the coming years will deliver new insights into many unanswered questions in mouse sex determination, and developmental biology more broadly, questions that remain critical to delivering advances in biomedicine and questions that fascinated Mary Lyon when she studied the genetics of the house mouse at Harwell (Lyon 1974; Bennett *et al.* 1975).

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