

## REVIEW

# Male sex determination: insights into molecular mechanisms

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Disorders of sex development often arise from anomalies in the molecular or cellular networks that guide the differentiation of the embryonic gonad into either a testis or an ovary, two functionally distinct organs. The activation of the Y-linked gene *Sry* (*sex-determining region Y*) and its downstream target *Sox9* (*Sry box-containing gene 9*) triggers testis differentiation by stimulating the differentiation of Sertoli cells, which then direct testis morphogenesis. Once engaged, a genetic pathway promotes the testis development while actively suppressing genes involved in ovarian development. This review focuses on the events of testis determination and the struggle to maintain male fate in the face of antagonistic pressure from the underlying female programme.

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## INTRODUCTION

Perceptions of sex and sexuality pervade modern culture. However, it is important to recognize that not all members of our society fit comfortably the socially constructed ideas of masculinity and femininity. These people are likely to struggle with a variety of medical and psychosocial issues surrounding their sexuality.<sup>1,2</sup> It is estimated that 1.7% of all live births have a disorder of sex development (DSD).<sup>3,4</sup> These conditions are congenital and are characterized by chromosomal or gonadal sex that does not match outward appearance of maleness or femaleness, or anatomical sex that is in some way ambiguous or intermediate between male and female.<sup>4</sup> Some of these conditions are associated with infertility, predisposition to gonadal tumours and/or other syndromic features.<sup>5,6</sup> Clearly, discovery of the underlying molecular causes of DSDs is an important goal in biomedical research.

Genomic and structure/function studies of human DSDs have revealed a number of genes as being important for sex development, while studies in the mouse have further extended our understanding of the mechanism of action of these genes; these approaches are complementary. Identifying the molecular mechanisms behind sex determination and differentiation will lead to more accurate diagnosis and prognosis, and assist in providing more informed options for psychological, endocrinological, surgical and other clinical management of DSDs, many of which remain uncharacterized at a molecular level. In a broader context, understanding the events of early testis development may also illuminate some of the underlying causes of male infertility.

In this review, we examine the molecular mechanisms behind male sex determination and differentiation, and how impairment of these mechanisms underlies a subset of human DSDs. In particular, we

highlight the interplay between the molecular pathways that promote male and female development, and the role of gene dosage and phenotype sensitivity in mice and humans.

## SRY AND THE BEGINNINGS OF MALENESS

We each inherit an X or a Y sex chromosome from our father and an X chromosome from our mother during fertilisation. The resulting chromosomal sex (XX or XY) leads to the transformation of the embryo into a male or a female. Before gonadal sex determination in both XX and XY embryos, a bipotential gonadal primordium exists that has the potential to differentiate into either testes or ovaries. Activation of the Y-linked gene *Sry* (*sex-determining region Y*) initiates testicular development. When *Sry* is expressed ectopically in XX mice, the testis pathway is initiated.<sup>7</sup> When *Sry* is not present, as in XX individuals, or non-functional in XY individuals, the bipotential gonads generally do not follow the testicular pathway and instead develop into ovaries.<sup>8,9</sup>

SRY plays a role in a number of DSDs: mutation or loss of function of SRY results in complete male to female sex reversal,<sup>10,11</sup> whereas ectopic expression of SRY in XX individuals due to chromosomal translocation of SRY may result in female to male sex reversal. Indeed, SRY translocation is responsible for 10% of all 46,XX female to male sex reversal.<sup>12</sup> Formation of ovotestes, where ovarian and testicular tissues coexist in the same organ, can also occur in cases of ectopic SRY activity.<sup>13,14</sup>

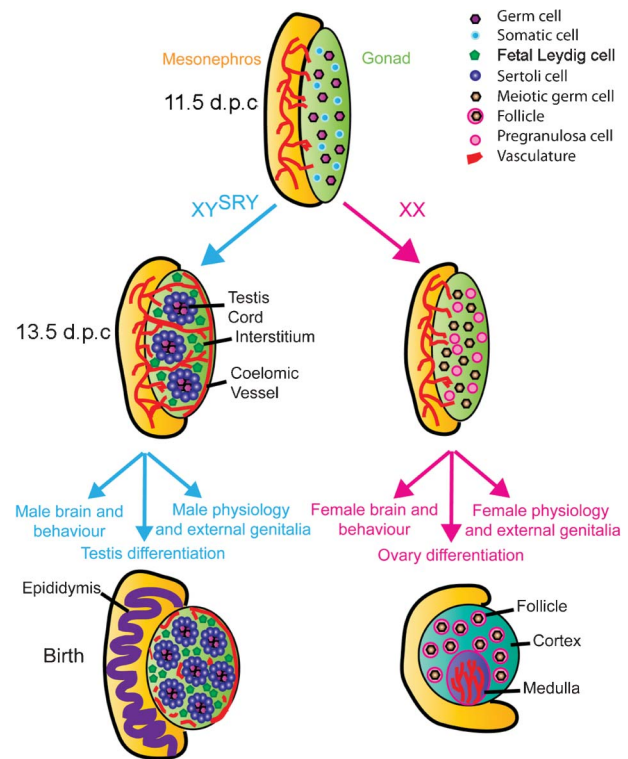
SRY is a transcription factor with a DNA-binding high-mobility group box domain.<sup>15,16</sup> In mice, expression of *Sry* is both brief and carefully regulated; however, the factors controlling this burst of expression remain unknown. One of the factors postulated to play a role in activation of *Sry* is Wilms' tumour 1 (WT1), which can act as a

transcriptional activator<sup>17,18</sup> or repressor.<sup>19</sup> WT1 has two active isoforms in the gonad, with either an insertion or an omission of three amino acids, lysine (K), tyrosine (T) and serine (S), between two zinc finger motifs.<sup>20,21</sup> Each isoform has distinct functions during mouse testis determination. Knockout mice show that WT1+KTS, although unlikely to be directly regulating *Sry* expression, is required for maintenance of the gonad.<sup>20</sup> However, this WT1 isoform has been shown to be able to transactivate the human *SRY* promoter *in vitro*.<sup>22</sup> Conversely, WT1+KTS-null mice exhibit complete XY sex reversal, presumably due to abnormally low *Sry* expression.<sup>20</sup> In agreement with the mouse model, WT1 haploinsufficiency, resulting in reduced levels of WT1+KTS, results in XY sex reversal in human patients.<sup>23</sup> It is proposed that WT1+KTS is involved in cell-autonomous regulation of *Sry* *in vivo*, as indicated by reduced *SRY* levels in cells of WT1+KTS-null mouse gonads.<sup>24</sup> It is important when interpreting these results to take into account the fact that knocking out one WT1 isoform leads to an increase in expression of the other, which may have an impact on the observed phenotype and interaction with *SRY*.

In addition to sufficiently early onset of expression of *Sry*, a threshold level of expression must be achieved for complete testis differentiation to occur. In mice, expression of *Sry* is initiated at 10.5 days post-coitum (d.p.c.), peaks at 11.5 d.p.c. and is extinguished by 12.5 d.p.c.<sup>25–27</sup> *Sry* expression occurs in a wave-like pattern, beginning in the central region of the gonad and expanding out towards the poles.<sup>28–31</sup> In humans, *SRY* has a broader spatiotemporal expression profile, occurring in multiple tissues such as the adrenal and heart, and being maintained for longer in the testis, apparently through to adulthood.<sup>32</sup> *Sry/SRY* is also expressed in the brain of mice and humans.<sup>32–34</sup> In mice, *SRY* is expressed in a subset of nigrostriatal dopaminergic neurons in the brain and appears to affect the specific motor behaviours they control.<sup>35</sup> However, although *SRY* is suspected of being involved in sexual dimorphism of the brain, a specific role for *SRY* outside sex determination has yet to be conclusively demonstrated.

Before turning to the cellular role of *SRY*, it is important to discuss briefly the course of events that occur as the testis differentiates. The gonads arise from a pair of bipotential primordia known as the genital ridges. In males, differentiation of the bipotential supporting cell lineage into Sertoli cells results in organisation of the developing testis into two main compartments: the testis cords, which comprise aggregates of germ cells surrounded by a layer of Sertoli cells in turn encased by peritubular myoid cells, and the testis interstitium which includes the steroidogenic Leydig cells and the testis vasculature. The development of secondary sexual characteristics in the embryo, such as external genitalia, is directed by the testes. Thus, the morphogenesis of the bipotential gonads into testes dictates the phenotypic sex of the male individual (Figure 1).

In mice, the first known cellular difference between the XX and XY gonad after expression of *Sry* is the male-specific proliferation of the epithelium at the coelomic surface of the genital ridges.<sup>36,37</sup> This sex-specific proliferation is thought to amplify the population of cells capable of differentiating into Sertoli cells, the first testicular cell type to differentiate,<sup>38</sup> and is required for the formation of testis cords.<sup>36,37,39</sup> Thus far, the molecular mechanism that induces coelomic epithelial proliferation is unknown. We do know a direct molecular target of *SRY*: the gene encoding the transcription factor *Sox9* (*Sry* box-containing gene 9).<sup>40</sup> Because testes develop normally in transgenic XX mice overexpressing *Sox9*, it appears that male-specific proliferation of the coelomic epithelium and all other aspects of foetal testis development are under the control, directly or indirectly, of *SOX9*.<sup>41,42</sup>



**Figure 1** Overview of mouse gonadogenesis. The expression of *Sry* and *Sox9* at 10.5–11.5 d.p.c. in the bipotential gonad initiates testis differentiation. By 13.5 d.p.c., basic testis morphology is established; the formation of testis cords, the coelomic blood vessel, and differentiation and activation of steroidogenesis in Leydig cells has occurred, and androgens are then produced by the testes. In the ovary, further differentiation is delayed. Around 13.5 d.p.c., germ cells have entered meiosis and vascularisation, and remodelling of the ovary to form germ cell cysts occurs. Later, the cortical and medullary domains begin to be established and folliculogenesis takes place. Secondary sexual characteristics include the establishment of the male and female genital tract and duct system, sex-specific brain dimorphisms and behaviours, and external genitalia. The establishment of secondary sexual characteristics involves organ-specific, regulatory gene networks. d.p.c., days post-coitum; FLC, foetal Leydig cell; Sox9, *Sry* box-containing gene 9; *Sry/SRY*, sex-determining region Y.

### SOX9 AND THE DIFFERENTIATION OF THE SERTOLI CELL

*Sox9* is upregulated when a protein complex of *SRY* and steroidogenic factor 1 ((SF1) nuclear receptor subfamily 5, group A, member 1) binds to a *Sox9* enhancer element known as testis-specific enhancer of *Sox9* core element (TESCO).<sup>40</sup> Like *SRY*, *SOX9* is necessary for testis differentiation: mice lacking *Sox9* undergo complete XY sex reversal,<sup>43,44</sup> while 75% of human patients with a heterozygous mutation in *SOX9* manifest with complete or partial XY sex reversal.<sup>45,46</sup>

The proposed mechanism for XY gonadal transcription of *Sox9* during sex determination consists of three distinct phases, according to Sekido and Lovell-Badge.<sup>40</sup> Firstly, SF1 initiates low-level transcription of *Sox9* in XX and XY genital ridges. Secondly, SF1, in a complex with *SRY*, activates male-specific transcription of *Sox9* in the male genital ridge *via* TESCO. High levels of *SOX9* are then maintained in the XY gonad *via* an autoregulatory feedback loop.<sup>40,47</sup> Indeed, *in vitro* studies using SF1, *SRY* and *SOX9* mutant proteins, modelled on clinical human mutations from 46,XY DSD patients, support this model. These proteins failed to activate the human homologue of TESCO, providing a potential mechanism by which mutations resulting in partially functional proteins can present as DSDs.<sup>48</sup>

It is believed that bipotential supporting cells cell-autonomously differentiate into Sertoli cells under the influence of SRY and SOX9: this conclusion was drawn from XX–XY chimaera studies in which it was found that, when testes formed, almost all Sertoli cells were XY, while other cell types did not exhibit a chromosomal bias.<sup>49,50</sup> However, some Sertoli cells were always found to be XX,<sup>49</sup> indicating the existence of paracrine pathways by which SRY- and SOX9-positive cells can recruit additional cells (such as XX cells in the chimaera experiments, or cells that express unusually low levels of *Sry* in normal XY gonads) to the Sertoli fate.

Two independent mechanisms of Sertoli cell recruitment are known: fibroblast growth factor 9 (FGF9) and prostaglandin D2 (PGD2) recruitment. Kim *et al.*<sup>51</sup> demonstrated using *Fgf9*-null mice, which exhibit XY sex reversal,<sup>52</sup> that FGF9 is required only for the maintenance of SOX9 expression, not its initiation. However, ectopic application of FGF9 to XX gonads induces SOX9 expression.<sup>51,53</sup> Hiramatsu *et al.*<sup>54</sup> showed that *Fgf9* expression occurs in a wave emanating from the central zone of the gonad similar to *Sry* and *Sox9*. Inhibition of FGF signalling repressed the expansion of the *Sox9*-positive domain in the XY gonad. Furthermore, removal of the central segment or isolation of the central domain of the testis before the expansion of *Fgf9* signalling resulted in failure of tubulogenesis in the anterior and posterior segments. Based on these findings, Hiramatsu *et al.*<sup>54</sup> proposed a system where FGF9 was produced in the central domain of the gonad by newly specified Sertoli cells, from which it was secreted and rapidly diffused towards the gonadal poles where it recruited cells to the Sertoli fate by reinforcing the expression of *Sox9*. This mechanism is supported by evidence from *Fgfr2*-null (FGF-receptor 2) mice whose phenotype is similar to *Fgf9*-null mice, displaying male-to-female sex reversal and suggesting FGFR2 is the receptor for FGF9 in the XY gonad. Indeed, conditional deletion of *Fgfr2* in pre-Sertoli cells shows that FGFR2 is required in pre-Sertoli cell differentiation.<sup>55</sup>

Independently, PGD2, an early product of the testis, is also able to induce Sertoli cell differentiation *in vivo* by amplifying SOX9 activity and canalizing the male pathway. Treatment of XX gonads with PGD2 resulted in upregulation in *Sox9* and its direct downstream target *Amh* (the gene encoding anti-Müllerian hormone), masculinizing the XX gonad.<sup>56–58</sup> However, *Pgds* (prostaglandin D2 synthase)-null testes, after a delay in Sertoli cell differentiation, develop normally, indicating that this mechanism is a nonessential backup system for Sertoli cell recruitment.<sup>56</sup> The requirement of these backup systems and the need to continually reinforce the male programme may stem from the weak but critical role of SRY and the need to actively suppress the underlying female programme. Existence of these recruitment mechanisms ensures that as many cells as required are pulled into the Sertoli fate to allow successful differentiation of the gonad.

### THE ROLE OF THE TESTIS VASCULATURE

Vascular patterning in the gonad is a sex-specific process.<sup>59</sup> Testis vasculature is formed by migration of endothelial cells into the developing testes.<sup>60,61</sup> By 12.5 d.p.c., a prominent artery known as the coelomic blood vessel can be seen along the anterior–posterior length of the testis, in addition to extensive microvasculature. Ectopic coelomic vessel-like structures were observed in gonads of XX mice mutant for *Rspo1* (*R-spondin* homologue 1), *Wnt4* (*wingless-related MMTV integration site 4*), *Fst* (*follicle-stimulating*) and *Ctnnb1* (*catenin (cadherin-associated protein), beta 1*).<sup>62–66</sup> *Rspo1* is a regulator of WNT4 signalling which involves *Ctnnb1*, while *Fst* is downstream of WNT4,<sup>67</sup> implicating the WNT signalling pathway in vessel formation and

patterning. Additionally, overexpression of *Wnt4* disrupts normal testis vasculature, indicating that WNT4 inhibits formation of gonad vasculature.<sup>68</sup> Notably, where testis vasculature is disrupted, as in the WNT4 overexpressing mice, Sertoli and foetal Leydig cells still differentiate.<sup>65,68</sup>

Recently, it has been discovered that the vascularisation of the testis plays an important instructive role in testis cord formation.<sup>60,61</sup> When endothelial migration was suppressed in testes by blocking vascular endothelial growth factors with VEGF-Trap or by using an antibody against vascular endothelial cadherin, testis cord morphogenesis was impaired.<sup>60,69</sup> Antagonizing vessel maturation also reduced proliferation of interstitial mesenchymal cells that appear to segregate the precursor territories for testis cords; this proliferation could be rescued by the addition of platelet derived growth factor isoform BB (PDGF-BB).<sup>61,69,70</sup> However, the mechanisms governing testis vascularisation and cord segregation are still unclear.

### LEYDIG CELLS: THE KEY TO PHENOTYPIC MASCULINISATION

Foetal Leydig cells (FLCs) produce steroid hormones that reinforce male-specific differentiation of the testis (for review, see Ref. 71). The FLC populations in humans and mice are similar during foetal life,<sup>72,73</sup> although observation of the induction of steroidogenesis largely limited to steroid level quantification. The origins of FLCs in humans remain unclear. In the mouse, FLCs arise by about 12.5 d.p.c. (for review, see Ref. 74), and recent work involving cell lineage tracing and live imaging suggests that they arise from multiple origins including the coelomic epithelium and the gonad/mesonephros border.<sup>53</sup> Several pathways and molecules have been implicated in their differentiation and maintenance. SF1 marks presteroidogenic and pre-Sertoli cells in the developing genital ridge<sup>75,76</sup> and acts as a key regulator of genes encoding steroid hydroxylases, which later distinguish FLCs.<sup>77</sup> Members of the hedgehog signalling pathway, desert hedgehog (DHH), which is secreted by Sertoli cells, and its receptor patched homologue 1 (PTCH1), which is expressed by the interstitium, have been shown to be positive regulators of FLC differentiation.<sup>78</sup> *Dhh*-null mice have a FLC differentiation defect.<sup>78</sup> Human patients with mutations in DHH present with mixed, partial or pure gonadal dysgenesis through to seemingly unaffected carriers.<sup>79–81</sup> In addition, chemical inhibition of hedgehog signalling at 11.5 d.p.c. completely abolished expression of steroidogenic enzymes, confirming that DHH/PTCH1 signalling is essential for FLC differentiation.<sup>78</sup> *Gli1* (*glioma-associated oncogene family zinc finger 1*) and *Gli2* (*glioma-associated oncogene family zinc finger 2*) are downstream targets of hedgehog signalling and are expressed exclusively in the testis interstitium in a manner similar to PTCH1.<sup>82</sup> However, *Gli1*- and *Gli2*-null mice display normal FLC differentiation, perhaps indicating functional redundancy between the GLI factors in FLCs.<sup>82</sup> Ectopic activation of the hedgehog signalling pathway in SF1-positive ovarian cells is sufficient to differentiate these cells into functional FLCs within an ovarian environment.<sup>83</sup> These ectopic cells upregulated SF1 and were able to partially masculinize the phenotype of the XX embryo.<sup>83</sup> Additionally, *Pdgr-α* (*platelet-derived growth factor receptor, alpha polypeptide*) and *Arx* (*Aristaless-related homeobox*) have been identified as being critical for FLC differentiation in knockout mouse models.<sup>84,85</sup>

### GERM CELLS: THE ORIGIN OF SPERM

Germ cells are the precursors of oocytes and spermatozoa in the foetal gonad. The sexual fate of the germ cell is determined by signalling factors that the germ cells are exposed to upon entry to the gonad,



rather than by their chromosomal constitution.<sup>58,86–89</sup> Much of what is known about the origin and regulation of the germ cells is derived from studies in mice, as discussed below.

In an ovary, germ cells must enter meiosis during foetal life if they are to initiate oogenesis correctly; conversely, meiosis must be avoided in male germ cells in the foetus if they are to embark on the spermatogenic pathway. The interplay between FGF9 and retinoic acid (RA) appears to be key to the correct specification of the germ cells in the mouse: meiosis is induced by RA in the foetal ovary and inhibited by FGF9, which is secreted by Sertoli cells, in the foetal testes.<sup>86–88,90</sup> In the developing testis, meiosis is avoided because RA is degraded by the P450 enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1).<sup>91</sup> Thus, CYP26B1 acts to suppress meiosis indirectly by the removal of RA, while FGF9 directly suppresses meiosis and acts to maintain pluripotency.<sup>86–88</sup> This mechanism is supported by *in vivo* evidence from *Cyp26b1*-null mice where degradation of RA does not occur in XY gonads, resulting in upregulation of RA-responsive *Stra8* (stimulated by retinoic acid gene 8) and germ cell entry into meiosis.<sup>87,91</sup>

Recently, a double-knockout of *Aldh1a2/Aldh1a3* (aldehyde dehydrogenase family 1, subfamily A2/A3), genes encoding key synthesizers of RA in the mesonephros, demonstrated that some meiosis still occurred in the foetal ovary.<sup>92</sup> These data indicate that either RA does not drive meiosis or, more likely, that there is an additional source of RA that remains in these mice. More in-depth analysis of this model will be required to clarify this point. Regardless, a strong antagonism exists between meiosis-promoting (female) factors and meiosis-suppressing (male) factors that push the resident germ cells into their respective fates.

Relatively, little is known about whether these mechanisms are used in humans. Culture experiments demonstrate that the RA initiates meiosis in the human ovary and can upregulate *STRA8*.<sup>93,94</sup> However, it appears that the human gonad has the capacity to produce RA, evidenced by the strong expression of *ALDH1A1* (aldehyde dehydrogenase family 1, subfamily A1) in the ovary around the time of meiosis initiation.<sup>93,94</sup> Most striking is the apparent lack of *CYP26B1* expression in the foetal human testes and the expression of RA receptors, indicating that the testes may be exposed to, and may be able to respond to, RA, unlike the situation in the mouse.<sup>93,95</sup>

Male germ cells are fated to enter G<sub>1</sub>/G<sub>0</sub> arrest in the foetal testes.<sup>89</sup> Retinoblastoma 1 is a cell cycle regulator necessary for male germ cells to enter arrest at the appropriate time. In XY *retinoblastoma 1*-null mice, the germ cell population fails to enter G<sub>1</sub>/G<sub>0</sub> arrest appropriately.<sup>96</sup> To compensate cell cycle suppressors, cyclin-dependent kinase inhibitors 1b and 2b are upregulated and after a delay can induce arrest.<sup>96</sup>

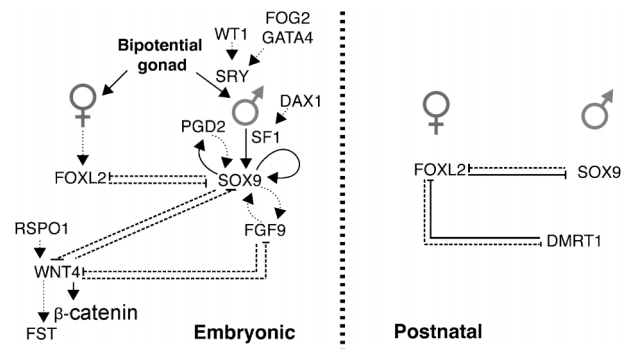
## OVOTESTES: WHAT THEY REVEAL ABOUT MALE–FEMALE ANTAGONISM IN THE EMBRYO

The study of ovotestes in mouse models has provided numerous insights into the antagonism between the male and female pathways during sex determination. When the Y chromosome derived from *Mus poschiavinus*, Y<sup>POS</sup>, is backcrossed to a C57/BL6 (B6) background, varying degrees of sex reversal are observed in the XY progeny.<sup>97</sup> This phenomenon is thought to be due to defective interaction between *Sry* on Y<sup>POS</sup> and autosomal sex-determining genes in B6.<sup>98</sup> Detailed expression studies have shown that a delay in the commencement of *Sry* expression, and subsequently, *Sox9* expression is the likely cause of B6-Y<sup>POS</sup> partial sex reversal.<sup>98–100</sup> Wilhelm *et al.*<sup>30</sup> found that although *Sry* was expressed throughout the genital ridge in B6-Y<sup>POS</sup> mice, the

upregulation of *Sox9* and activation of downstream testis differentiation pathways only took place in the central zone where *Sry* expression was initiated.<sup>25,28,29,31</sup> These findings indicate that expression of *Sry* in the poles, which in ovotestes differentiate into ovarian tissue, does not reach the required expression threshold early enough. This failure to upregulate *Sox9* in pre-Sertoli cells allows the expression of key ovarian differentiation genes and the engagement of the ovarian programme. Therefore, male–female antagonism underlies the successful differentiation of the gonad. When the balance of factors is altered, even slightly, the underlying battle between the testicular and ovarian fate is revealed.

## MOLECULAR BALANCING ACTS: EXAMPLES OF ANTAGONISM BETWEEN MALE AND FEMALE PATHWAYS

In order to understand the molecular mechanisms behind male sex determination, we must also understand what is occurring molecularly in the antagonistic female programme at the time of sex determination. It has become clear that the ovarian programme, although it is considered the ‘default’, is an active genetic programme in its own right.<sup>101</sup> The antagonism between the male and female pathways has been illustrated using genetic approaches (Figure 2). In an early study of male–female antagonism, the male pathway was suppressed by knocking out *Fgf9*; as a result, the female pathway was promoted, indicated by the upregulation of *WNT4*.<sup>51</sup> Conversely, when the female pathway was suppressed by knocking out *Wnt4*, the male pathway was stimulated.<sup>102</sup> This phenomenon correlates to cases in DSD patients where *WNT4* loss or mutation results in XX masculinisation and duplication results in XY feminisation.<sup>68,103</sup> This male–female antagonism is further supported by *ex vivo* work. Treatment of XX gonads with ectopic FGF9 suppressed normal *WNT4* expression and induced ectopic upregulation of *Sox9*.<sup>51</sup> Thus, readouts of the male



**Figure 2** Model of molecular interactions and proposed levels of antagonism in testis and ovary. For details see text. In the embryonic XY gonad, *Sry* is activated via a mechanism involving WT1 (indirect regulation illustrated by dotted arrow/line). *SRY* then directly upregulates *Sox9*, direct regulation illustrated by solid arrow/line which maintains its own expression; positive feedback loops exists between PGD2/*Sox9* and FGF9/*Sox9*. In the XX gonad, FOXL2 is active as are RSP01, β-catenin and WNT4, which is required for expression of *Fst*. During embryonic development, antagonism is thought to exist between FGF9/WNT4, *Sox9*/WNT4 and *Sox9*/FOXL2. Postnatally, antagonism exists between *Sox9*/FOXL2 and DMRT1/FOXL2. DAX1, Nr0b1, nuclear receptor subfamily 0, group B, member 1; DMRT1, doublesex and mab-3-related transcription factor 1; FGF9, fibroblast growth factor 9; FOG2, ‘Friend of Gata1’ type 2; FOXL2, forkhead box L2; Fst/FST, follistatin; GATA4, GATA-binding protein 4; PGD2, prostaglandin D2; RSP01, R-spondin homologue 1; SF1, steroidogenic factor 1; *Sox9*/*Sox9*, *Sry* box-containing gene 9; *Sry*/*SRY*, sex-determining region Y; WNT4, wingless-related MMTV integration site 4; WT1, Wilms’ tumour 1.

pathway, SOX9 and FGF9 can increase in response to a weakening of the female programme and *vice versa*.

Recent studies have shown that the balance between the antagonistic testicular and ovarian differentiation programmes is also important postnatally. Gonad-specific transcription factors doublesex and mab-3-related transcription factor 1 (DMRT1) and forkhead box L2 (FOXL2) have roles in maintenance of the testes and ovaries respectively.<sup>104</sup> In humans, *DMRT1* hemizygoty may result in hypogonadism, often with streak gonads.<sup>105</sup> *FOXL2/FoxL2* is a key ovarian marker, and its mutation is associated with premature ovarian failure in human patients and knockout mice.<sup>104,106,107</sup> *Dmrt1*-null males were found to have numerous FOXL2-positive cells in the seminiferous tubules 4 months after birth.<sup>108</sup> A Sertoli cell-specific knockout of *Dmrt1* confirmed that loss of DMRT1 in Sertoli cells and not germ cells allows ectopic FOXL2 expression.<sup>108</sup> However, FOXL2 may also be able to repress DMRT1, as shown by strong upregulation of DMRT1, when FOXL2 is ablated postnatally,<sup>109</sup> suggesting the existence of a mutual antagonism necessary for maintaining sex differentiation throughout life.

FOXL2 has also been shown to antagonize SOX9 in the adult ovary and is postulated to also play a role in the embryonic testes. Conditional deletion of *FoxL2* at 8 weeks resulted in transdifferentiation of granulosa cells in the adult ovary to Sertoli-like cells expressing SOX9 even in the presence of oocytes and theca cells, the steroidogenic cells of the ovary, differentiated into Leydig-like cells.<sup>109</sup> In the adult ovary, it was demonstrated using chromatin immunoprecipitation that FOXL2 can repress TESCO activity,<sup>109</sup> providing a possible mechanism for the role of FOXL2 in maintaining ovarian function postnatally. *FoxL2* is also upregulated in XY embryonic gonads of *Sox9*-conditional null mice indicating that a similar mechanism exists during embryonic sex differentiation.<sup>43</sup> However, importantly, expression of male and female factors in the gonad is exclusive, as observed in the ovotestes,<sup>30</sup> such that FOXL2 and SOX9 are never coexpressed in the same cell. Nevertheless, while the factors mentioned in the above section are responsive to each other's loss, direct interaction and antagonism between any of these factors is yet to be demonstrated.

### SF1: HIGHLIGHTING THE ROLE OF GENE DOSAGE DIFFERENCES IN THE MOUSE AND HUMAN

SF1 is a striking example of a factor whose effects on gonadal phenotype are sensitive to dosage and genetic background.<sup>75</sup> *Sf1*-null mice undergo early gonadal and adrenal development, but the organs regress by 11.5 d.p.c.<sup>75,110</sup> Transgenic expression of SF1 in *Sf1*-null mice was able to rescue gonad development, but not adrenal development.<sup>111</sup> Additionally, *Sf1*-haploinsufficient mice have disrupted adrenal development but develop testes normally.<sup>112</sup> Together, these results indicate that SF1 is involved in gonad and adrenal organ maintenance and that, in the mouse, the adrenal is more sensitive to *Sf1* dosage than the gonad. However, there is evidence that the reverse is true in humans. Two patients with heterozygous mutations in *SF1* have been shown to exhibit gonadal dysgenesis but normal adrenal function, indicating that, in humans, testis development is more sensitive to *Sf1* dosage than adrenal gland development.<sup>113,114</sup>

### DAX1: A QUESTION OF GENE DOSAGE AND GENETIC BACKGROUND

*Dax1* (*Nr0b1*, nuclear receptor subfamily 0, group B, member 1) expression in the mouse indicates a role in early sex determination, with *Dax1* being expressed in both sexes initially before being downregulated in the testes.<sup>31,115</sup> Duplications of chromosomal region Xp21.2–21.1,

which includes *DAX1*, are sufficient to impair testis differentiation in 46,XY human patients and induce male-to-female sex reversal (dosage-sensitive sex reversal).<sup>116–118</sup> These data imply that *DAX1* acts as an antitestis gene, antagonizing the action of SRY. Similarly, overexpression of *Dax1* in mice can induce male-to-female sex reversal. However, only mice highly overexpressing *Dax1* and which also have a weakened male sex-determining pathway driven by a  $Y^{POS}$  chromosome, show complete male-to-female sex reversal.<sup>31</sup> A common interpretation of these data is that mice and humans may have different sensitivity thresholds for *DAX1* dosage.

An alternative explanation is that genetic background and the presence of genetic modifiers can explain whether or not gonad sex reversal occurs. Genetic background is a key determinant in the penetrance of a number of sex reversal phenotypes, with heterozygous mutants often displaying different phenotypes depending on their genetic background. Bouma *et al.*<sup>119</sup> compared sex development in *Gata4* (GATA-binding protein 4)<sup>120,121</sup> and *Fog2* ('Friend of Gata1' type 2; Zfp2, zinc finger protein, multitype 2)<sup>120,121</sup> heterozygous mutants on B6/ $Y^{AKR}$ , B6, D2/ $Y^{AKR}$  and D2 backgrounds, which have decreasing sensitivity to sex reversal. They found that ovaries or ovotestes developed in these mutants, but only on the B6/ $Y^{AKR}$  background. All other strains developed testes normally. These studies demonstrate the importance of genetic background, and also clearly indicate a role for GATA4 and FOG2 in foetal testis development. A similar background dependence was observed in the generation of sex-reversed *Dax1* mutant mice.<sup>31,122–124</sup> Given that genetic background and the presence of genetic modifiers can explain whether or not gonad sex reversal occurs, it follows that effects attributed to differences in dosage sensitivity between humans and mice might also be explained by genetic background.

Given the expectation that *Dax1* acts as an antitestis gene, deletion of *Dax1* in mice produced a surprising result: ovaries formed normally in *Dax1*-null XX mice, but in XY mice with a deletion of *Dax1*, testis cord formation was abnormal.<sup>123</sup> Moreover, in XY *Dax1*<sup>-/-</sup>/ $Y^{POS}$  mice, complete male-to-female sex reversal was observed.<sup>124</sup> Subsequent studies revealed the cause of the male-to-female sex reversal: *Sox9* is not upregulated in XY *Dax1*-null mice, despite normal expression of *Sry*.<sup>125</sup> Nonetheless, strong overexpression of *Sry* in XY<sup>POS</sup> *Dax1*-null mice was able to upregulate *Sox9*, correcting testis development and producing fertile males.<sup>125</sup> These results are somewhat in accord with findings in humans, with respect to *DAX1* mutations. 46,XY patients with deletion of, or mutations in, *DAX1*, exhibit the male phenotype, but have hypogonadotrophic hypogonadism and disorganized testis cords, indicating that *DAX1* is not essential for the initial stages of human gonad development, but is required for testis development.<sup>117,124,125</sup> As there is evidence that *DAX1* can work in both 'antitestis' and 'protestis' capacities, it is possible that correct function in the testis occurs only within a 'window' of activity involving two concentration thresholds. In this scenario, *DAX1* activity beyond an upper threshold, for example, in cases of *DAX1* duplication, may act to antagonize testis differentiation, while *DAX1* activity below a lower threshold, for example, in cases of mutation, may allow ovarian differentiation to occur.<sup>126</sup>

### CONCLUSIONS AND PERSPECTIVES

From the above discussion, it is clear that initial specification of Sertoli cells is a result of SRY expression, with the effects of SRY mediated largely if not entirely by SOX9. A complete understanding of male sex determination and testis morphogenesis, therefore, largely depends on a deeper understanding of the molecular and

cellular roles of SOX9, and therefore, on the discovery and characterisation of all transcriptional targets of SOX9. Further, it remains to be clarified how the Sertoli cell directs events during morphogenesis; that is, what signalling molecules are produced by these cells and how they influence the differentiation of the other testicular cell lineages. How the other cell lineages, once masculinized, then contribute to testis morphogenesis is also a question that requires further investigation. A greater understanding of the molecular interactions involved in the process of testis differentiation will provide new avenues for DSD diagnosis and management.

Commitment to the male fate and then maintenance of that fate is achieved only by overcoming the progress of the female pathway, and antagonistic interplay is seen during foetal life, as well as postnatally. Mutual antagonism is likely to be facilitated by transcription factors such as SOX9 and FOXL2, but assays to determine the molecular interactions that underpin these antagonistic relationships have yet to be completed. Understanding what factors facilitate antagonism between the male and female programmes will lead to a greater understanding of what pushes individuals into or out of the two typical sex phenotypes specified by XX and XY chromosomes.

Additionally, as demonstrated here, sex-determining genes often respond in a dosage-dependent manner that may also be influenced by genetic background. As a result, human DSDs and mouse models may not always phenocopy each other, highlighting the need for a collaborative approach to DSD identification and diagnosis between researchers and clinicians. Human patient cases demonstrate, especially, that gene dosage and genetic background can be important factors in phenotype severity. However, despite this, the investigation of gene dosage effects and the effects of genetic background on sex determining genes in the mouse is a question that few researchers have addressed and that warrants further detailed investigation. Tools, such as inducible knockout and single-copy transgenesis strategies, are now available to study these phenomena in the mouse. Thus, better integration of lessons from both human cases and mouse models must be a priority. Finally, it is clear that integration of clinical findings and mouse models will contribute to a better understanding not only of the causes of human DSDs but also of the basic biology of sex determination in the male.

**Abbreviations:** DSD, disorder of sex development; DNA, deoxyribonucleic acid; HMG, high-mobility group-box; WT1/*WT1*/*Wt1*, Wilms Tumor 1; K, lysine; T, tyrosine; S, serine; *Sry*, sex determining region of Chr Y; SOX9/*SOX9*/*Sox9*, SRY-box containing gene 9; SF1/*SF1*/*Sfl* (Nr5a1), steroidogenic factor 1 (nuclear receptor subfamily 5, group A, member 1); TESCO, testis-specific enhancer of *Sox9* core element; FGF9/*FGF9*/*Fgf9*, fibroblast growth factor 9; PDG2/*PDG2*/*Pdg2*, prostaglandin D2 synthase; FGF, fibroblast growth factor; FGFR2/*FGFR2*/*Fgfr2*, Fgf-receptor 2; AMH/*AMH*/*Amh*, anti-Mullerian hormone; PGDS/*PGDS*/*Pgds*, prostaglandin D2 synthase; dpc, days post coitum; WNT4/*WNT4*/*Wnt4*, wingless-related MMTV integration site 4; FLC, fetal Leydig cell; DHH/*DHH*/*Dhh*, desert hedgehog; PTCH1/*PTCH1*/*Ptch1*, patched homolog 1; GLI1/2/*GLI1*/2/*Gli1*/2, glioma-associated oncogene family zinc finger 1/2; PDGFR- $\alpha$ /*PDGFR- $\alpha$* /*PDGFR- $\alpha$* , platelet derived growth factor receptor, alpha polypeptide; ARX/*ARX*/*Arx*, aristaless related homeobox; RA, retinoic acid; STRA8/*STRA8*/*Stra8*, stimulated by retinoic acid gene 8; RB1/*RB1*/*Rb1*, retinoblastoma 1; Cdkn1b/2b/*Cdkn1b*/2b/*Cdkn1b*/2b, cyclin dependent kinase inhibitors 1b/2b; CYP26B1/*CYP26B1*/*Cyp26b1*, cytochrome P450, family 26, subfamily b, polypeptide 1; Aldh1a1/2/3/*Aldh1a1*/2/3/*Aldh1a1*/2/3, aldehyde dehydrogenase family 1, subfamily A1/A2/A3; Y<sup>POS</sup>, Y chromosome derived from *Mus poschiavinus*;  $\beta$ -catenin/*CTTNB1*/*Ctnnb1*, catenin (cadherin associated

protein), beta 1; FOXL2/*FOXL2*/*FoxL2*, forkhead box L2; DMRT1/*DMRT1*/*Dmrt1*, doublesex and mab-3 related transcription factor 1; FOG2/*FOG2*/*Fog2*, *Zfp*m2, zinc finger protein, multitype 2; GATA4/*GATA4*/*Gata4*, GATA binding protein 4; DAX1/*DAX1*/*Dax1*, Nr0b1, nuclear receptor subfamily 0, group B, member 1; FST/*FST*/*Fst*, follistatin; RSPO1/*RSPO1*/*Rspo1*, R-spondin homolog; PDGF-BB, platelet derived growth factor isoform BB.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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