Genetic regulation of mammalian gonad development

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Abstract | Sex-specific gonadal development starts with formation of the bipotential gonad, which then differentiates into either a mature testis or an ovary. This process is dependent on activation of either the testis-specific or the ovary-specific pathway while the opposite pathway is continuously repressed. A network of transcription factors tightly regulates initiation and maintenance of these distinct pathways; disruption of these networks can lead to disorders of sex development in humans and male-to-female or female-to-male sex reversal in mice. *Sry* is the Y-linked master switch that is both required and sufficient to drive the testis-determining pathway. Another key component of the testis pathway is *Sox9*, which acts immediately downstream of *Sry*. In contrast to the testis pathway, no single sex-determining factor has been identified in the ovary pathway; however, multiple genes, such as *Foxl2*, *Rspo1*, *Ctnnb1*, and *Wnt4*, seem to work synergistically and in parallel to ensure proper ovary development. Our understanding of the regulatory networks that underpin testis and ovary development has grown substantially over the past two decades.

Eggers, S. *et al. Nat. Rev. Endocrinol.* **10**, 673–683 (2014); published online 23 September 2014; doi:10.1038/nrendo.2014.163

Introduction

Sexual reproduction in mammals necessitates two sexes, each of which is characterized by sex-specific anatomical and genetic features. The phenotypic sex of an individual is largely governed by the type of gonad that develops in the embryo, a process that is determined by an individual's chromosome complement (specifically the presence or absence of a Y chromosome). Once the gonads have developed they begin producing sex-specific hormones, androgens in male and estrogens in female individuals, which in turn determine the development of secondary sexual characteristics, such as the external genitalia. This process is also known as sex differentiation.[1] In addition, the structure of these two distinct organs, as well as the type of sex hormones produced, influences the production and maturation of germ cells (sperm and oocytes). During early development the embryonic gonad is bipotential with the unique ability to differentiate into one of two functionally distinct organs, a testis or an ovary. Proper determination, differentiation and development of the gonads rely on a tightly regulated network of transcription factors and cell signalling molecules. Disruption of the genetic network that underlies these pathways can lead to disorders of sex development (DSDs), which are associated with gonadal dysfunction, infertility and gonadal cancer in affected individuals. DSDs comprise a wide spectrum of phenotypes, such as hypospadias in male patients and ambiguous genitalia or complete sex reversal in both male and female individuals. The phenotypic variation associated with DSDs is a consequence of genetic lesions that can disrupt any one of the multiple elements in the regulatory network that dictates gonadal specification and development.

In this Review, the key genes involved in mouse testis and ovary development are examined with a focus on findings from experimental models of gonad development. Over the past few years, novel elements in previously characterized pathways and new levels of gene regulation, such as noncoding RNAs[2] and epigenetics,[3] have been implicated in gonad development. Here, we focus on the genes required for the structural development and maintenance of the gonads. The effects of steroidogenesis and hormones as components of gonadal development are beyond the scope of this Review.

Formation of the bipotential gonad

During mouse embryonic development, the bipotential gonad first appears at embryonic day (E)10.5. Several transcription factors are critical to the development of the undifferentiated bipotential gonad during embryogenesis (Figure 1; Table 1). Mutations in the genes that encode these transcription factors can lead to development of streak gonads, structures that are characterized by an abundance of nonfunctional fibrous tissue and that fail to develop much beyond the embryonic bipotential state.[4]

Homeobox protein Emx2 and Lim/homeobox protein Lhx9 (encoded by *Emx2* and *Lhx9*, respectively) are involved in early development of the bipotential gonad. *Emx2* encodes a homeodomain transcription factor with a critical role in early development of the urogenital and central nervous systems. Mice deficient in *Emx2* completely lack gonads, kidneys and genital tracts, a finding that supports a key role for this protein in the development of the early urogenital system and the bipotential

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Competing interests
The authors declare no competing interests.

Key points

- The critical testis-determining genes are *Sry* and *Sox9*; however, many genes upstream of *Sry* and downstream of *Sry* and *Sox9* are also important for correct testis development
- Novel factors have been identified in the known pathways of the testis-determining network, including *Six1, Six4*, *Map3k4*, *Gadd45g* and *Hhat*
- Ovarian development lacks a single genetic switch; however, genes such as *Rspo1*, *Foxl2, Wnt4* and *Ctnnb1* seem to be essential for correct ovary development
- Both the testis-determining and ovary-determining pathways have active gene networks that must be maintained throughout life by suppression of the opposing pathway
- As well as regulation of gene expression by transcription factors, other types of gene control, such as noncoding RNAs and epigenetic modification, are required for gonad development

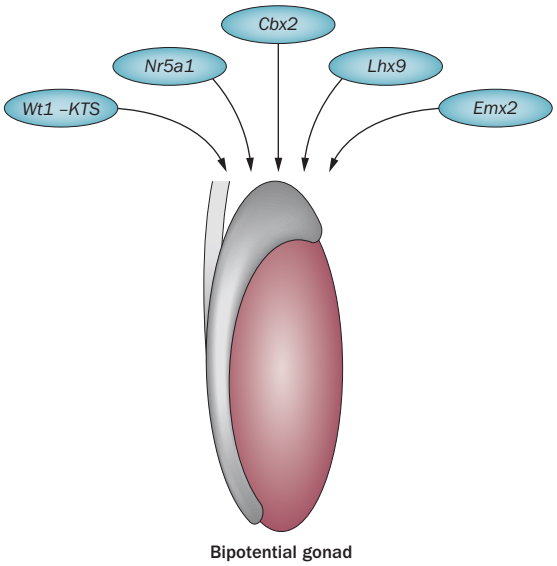

Figure 1 | Genes and pathways required for the development of the bipotential gonad. Genes essential for development of the bipotential gonad have been identified by specific knockout mouse strains that show a total absence of gonad development. Functional studies revealed that the Wt1 –KTS isoform binds to and activates the *Nr5a1* promoter in conjunction with Lhx9. In the spleen and adrenal gland, Cbx2 has been shown to regulate *Nr5a1*, suggesting that a similar association occurs in the bipotential gonad. Emx2 is thought to have a role in bipotential gonad development through proliferation, differentiation and/or cell survival pathways.

gonad.[5] *Lhx9*-null mice also fail to develop gonads, and XY mice that are deficient in *Lhx9* develop as female mice phenotypically due to the absence of testosterone and Müllerian-inhibiting factor (also known as anti-Müllerian hormone).[6] As Lhx9 regulates expression of *Nr5a1* (a key gene required for very early gonad development),[6,7] this protein is likely to be positioned near the top of the transcription factor network.

Nr5a1 encodes the orphan nuclear receptor Nr5a1 (also known as steroidogenic factor 1), a transcription factor that is expressed in the gonads and in all primary steroidogenic tissues, including the adrenal glands.[8,9] *Nr5a1*-null mice lack both gonads and adrenal glands,[9] which demonstrates an essential role of this gene in gonad development and steroidogenesis.[8,9] Nr5a1 also plays an important part in upregulating expression of the testis-determining gene, *Sox9*.[10]

Anti-Müllerian factor (encoded by *Amh*) is one of the earliest hormones produced by the testis and is responsible for regression of the female Müllerian ducts.[7] Transcriptional control of *Amh* is regulated by Nr5a1 in cooperation with the –KTS isoform of Wilms tumor protein homolog (Wt1),[11] a transcription factor necessary for early gonad development and subsequent testis differentiation. As with other transcription factors expressed during early gonad and urogenital development, *Wt1*-null mice fail to develop gonads and kidneys, which supports a very early role for this gene in formation of the bipotential gonads.[12] Another Wt1 isoform, +KTS, seems to have an important function very early in testis development. Mice lacking Wt1 +KTS undergo male-to-female sex reversal as a consequence of failure to upregulate expression of *Sry*.[13]

An additional gene involved in formation of the bipotential gonad is *Cbx2*. Mice deficient in *Cbx2* display retarded gonad development and male-to-female sex reversal.[14] *Cbx2* is also involved in the upregulation of several testis genes, including *Nr5a1*, *Wt1* and *Sry*.[15]

Testis determination

Sox transcription factors

The *Sox* (*Sry*-related HMG box) genes encode a family of transcription factors involved in the development of a variety of tissues and cell lineages, such as the vasculature (Sox17, Sox18) and the skeleton (Sox9, Sox5, Sox6).[16] Sox transcription factors bind to the minor groove structures of DNA, which causes the DNA to bend, thereby permitting cofactors (transcriptional activators or repressors) to bind to the promoter or regulatory regions of target genes. Thus, Sox proteins can modulate expression of downstream target genes to ensure correct specification, differentiation and maintenance of different cell types and tissues (Figure 2; Table 2).[17,18]

The first *Sox* gene family member to be identified was *Sry* (which encodes the sex-determining region Y protein).[19–22] In mice and humans, *Sry* is both necessary and sufficient to induce testis development.[19,21] When expression levels of *Sry* reach a critical threshold, expression of its downstream target, *Sox9*, is activated.

In both male and female developing mouse embryos, *Sox9* is expressed in the genital ridge at E10.5; however, *Sry* expression peaks at E11.5 in XY embryos and, as a consequence, expression of *Sox9* becomes sexually dimorphic.[23,24] In XY gonads, *Sox9* expression is considerably increased, which is in stark contrast to XX gonads where *Sox9* expression is decreased. Only *Sry*-positive cells in XY gonads express high levels of *Sox*9 and subsequently differentiate into Sertoli cells.[25,26] Conditional, gonad-specific knockout of *Sox9* (using the Cre–LoxP system) in XY embryos led to the development of ovaries[27,28] and ectopic overexpression of *Sox9* in XX embryonic gonads (using a *Wt1–Sox9* transgene, integrated into the genome via homologous recombination)

Table 1 | Genes involved in regulation of bipotential gonad development

Gene (protein)	Protein function	Gonad phenotype in mouse models	Reference
Emx2 (empty spiracles homeobox 2)	Transcription factor	*Emx2*$^{-/-}$ mice lack kidneys, ureters, gonads and genital tracts	Miyamoto *et al.* (1997)[5]
Cbx2 (chromobox homolog 2)	Transcription factor	*Cbx2*$^{-/-}$ XY male-to-female sex reversal and XX impaired ovary development	Katoh-Fukui *et al.* (1998)[14]
Lhx9 (LIM homeobox 9)	Transcription factor	*Lhx9*$^{-/-}$ mice fail to develop a bipotential gonad	Birk *et al.* (2000)[6]
Nr5a1 (nuclear receptor subfamily 5, group A, member 1 [steroidogenic factor 1; Sf1])	Nuclear receptor Transcription factor	*Nr5a1*$^{-/-}$ mice fail to develop a bipotential gonad	Luo *et al.* (1994)[9]
Wt1 (Wilms tumor 1)	Transcription factor	*Wt1*$^{-/-}$ mice fail to develop a bipotential gonad	Kreidberg *et al.* (1993)[12]

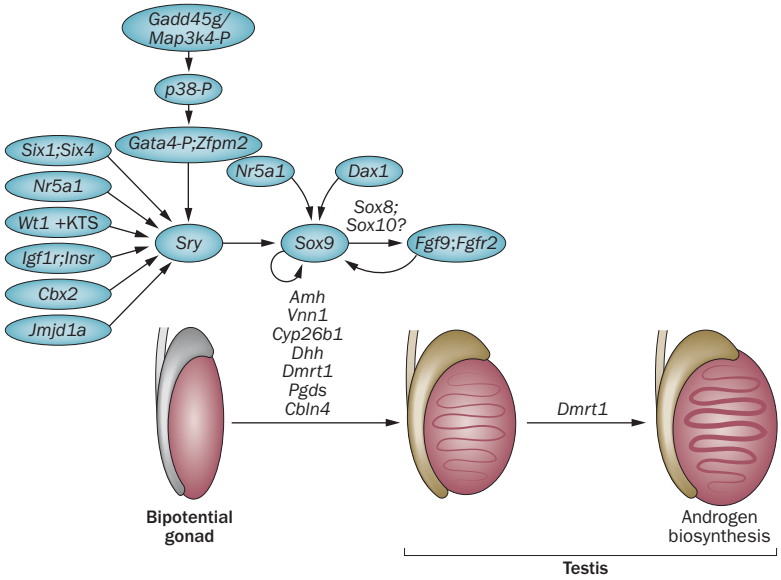

Figure 2 | Genes and pathways required for testis development and differentiation. Genes such as *Nr5a1*, *Six1, Six4*, *Wt1* (+KTS isoform), *Igf1r, Insr*, *Cbx2*, *Jmjd1a*, and the Gadd45g–Map3k4P–p38P–Gata4–Zfpm2 (also known as Fog2) pathway are essential for the correct initiation and upregulation of *Sry*. In XY mouse embryos, *Sry* is transiently expressed in the bipotential gonad, reaching its highest expression levels at E12.5. Sry expression initiates an increase of *Sox9* expression in the developing testis, which then stimulates *Fgf9* expression. Both Fgf9 and Sox9 act in a positive feedback loop to further upregulate Sox9. In addition to regulating *Sry*, Nr5a1 is also involved, together with Dax1 (also known as Nr0b1), in regulation of *Sox9* expression. Numerous other genes, such as *Sox8–Sox10*, *Fgfr2*, *Amh*, *Vnn1*, *Cyp26b1*, *Dhh*, *Pgds* and *Cbln4*, and their gene products, are necessary for the regulation and maintenance of this crucial testis pathway. *Dmrt1* is required for the maintenance of the differentiated testis and is also crucial for preventing female (ovary) reprogramming in postnatal mouse testis. Abbreviation: P, phosphate.

resulted in development of testes.[29,30] Interestingly, Sertoli-cell-specific ablation of *Sox9* in mice (after E13.5) resulted in normal testis development and XY mice with Sertoli-specific *Sox9* deficiency are fertile until approximately 5 months of age.[31] This phenotype might reflect functional redundancy amongst the *Sox* gene family members; consequently, *Sox9* deficiency in Sertoli cells might be functionally rescued by either of two related *Sox* genes, *Sox8* or *Sox10*, which are also expressed in the testis during gonadogenesis.

Several studies have reported tissue-specific regulatory elements of *Sox9* in the gene desert upstream of this gene, including elements required for the expression of *Sox9* in skeletal and craniofacial development. In human patients, rearrangements of the region upstream of *SOX9* lead to skeletal malformations (campomelic dysplasia, with or without a DSD, depending on the region affected) and craniofacial malformations (Pierre Robin syndrome, which is frequently part of campomelic dysplasia).[32] Studies in mice examined sex reversal phenotypes that could also be ascribed to the disruption of the upstream regulatory region of *Sox9*. Some of these studies focused on *Oddsex* mice, which carry a 150 kb deletion located 1.3 Mb upstream of the *Sox9* gene[29,33] that perturbs transcriptional regulation, either by disrupting a testis-specific regulatory element or by altering the three-dimensional looping (chromosome conformation) and interaction of multiple regulatory elements of *Sox9*, thus resulting in male-to-female sex reversal. Another study identified a testis-specific enhancer of *Sox9* located just upstream of the translational start site of *Sox9*, but it should be noted that this region is some Mb away from the *Oddsex* region. This study showed that, in mice, synergistic binding of Nr5a1 and Sry to the testis-specific enhancer core element (TESCO), located ~13 kb upstream of *Sox9*, is required for upregulation of *Sox9* expression. Once the Sox9 protein is present, it displaces Sry from the regulatory element and autoregulates its own expression.[10]

Another, study identified a far-upstream (~70 kb) enhancer of *Sox9* (SOM), which is required for the autoregulation of *Sox9* expression in distinct somatic tissues, such as the pancreas, lung and kidney, but not in testis and heart valves.[34] The study researchers postulated that, although Sox9 can control its own regulation in testis[10] as well as in the above-mentioned somatic tissues, it might do so using distinct enhancers and cofactors and a different synergistic action of regulatory elements. The researchers also postulated that Sox9 binds as a dimer to SOM and as a monomer to TESCO.

Although the mechanisms by which *Sox9* expression is regulated have been extensively characterized, the complete complement of downstream targets of Sox9 remains largely unknown. Mouse models of gonadal development indicate that Sox9 initiates expression of the gene encoding the key signalling molecule fibroblast growth factor 9 (*Fgf9*), as well as other downstream targets, such as *Amh*,[35] *Vnn*,[36] *Pgds*,[37] and *Cbln4*.[38] Additionally, other genes have been identified downstream of *Sry* and *Sox9*, which are potential targets of these transcription factors, especially those that are upregulated in the male gonad during the narrow developmental window between peak expression of Sry (E12.5) to the start of testis migration (E14.5) For many of these genes, however, evidence is still insufficient to implicate them as genuine downstream targets.

Table 2 | Genes involved in regulation of testis development

Gene (protein)	Protein function	Gonad phenotype in mouse models	Reference
Dhh (Desert hedgehog)	Signalling molecule	*Dhh*$^{-/-}$ mice have disruption of testis cord formation owing to abnormal peritubular tissue	Bitgood *et al.* (1996)[75]
Dmrt1 (Doublesex and Mab3 related transcription factor 1)	Transcription factor	*Dmrt1*$^{-/-}$ mice have impaired testis development at postnatal day 2 and loss of Sertoli and germ cells Postnatal knockout of *Dmrt1* in Sertoli cells leads to transdifferentiation of testes into ovaries	Raymond *et al.* (2000)[98] Matson *et al.* (2011)[90]
Fgf9 (fibroblast growth factor 9)	Signalling molecule	*Fgf9*$^{-/-}$ XY mice have male-to-female sex reversal and impaired development of Sertoli cells	Kim *et al.* (2006)[94]
Gadd45g (growth arrest and DNA damage-inducible protein γ)	Nuclear protein Protein–protein interactions suggest a cofactor role	XY male-to-female sex reversal on B6 backgrounds (C57BL/N[61] and C57BL/6J[62]) No gonadal phenotype on mixed background[63]	Gierl *et al.* (2012)[63] Warr *et al.* (2012)[64] Hoffmeyer *et al.* (2012)[65]
Gata4 (GATA-binding factor 4)	Transcription factor	*Gata4*$^{-/-}$ mice have embryonic lethality (E7–E9.5) *Gata4*^{ki} mice have severe anomalies of testes	Tevosian *et al.* (2002)[48]
Hhat (Hedgehog acyl-transferase)	Membrane-bound acyltransferase	*Hhat*$^{Creface/Creface}$ XY mice have gonadal dygenesis and skeletal and growth defects	Callier *et al.* (2014)[80]
Kdm3a, also known as *Jmjd1a* (lysine-specific demethylase 3A)	Zinc finger protein Histone demethylase	XY male-to-female sex reversal with variable gonadal phenotypes; frequency of sex reversal is dependent on origin of the Y chromosome	Kuorki *et al.* (2013)[3]
Map3k1 (mitogen-activated kinase kinase kinase 3)	Kinase	No gonadal phenotype on mixed genetic background Minor abnormalities on C57BL/6J background	Warr *et al.* (2011)[99]
Map3k4 (mitogen-activated kinase kinase kinase 4)	Kinase	XY male-to-female sex reversal	Bogani *et al.* (2009)[61]
Nr0b1, also known as *Dax1* (nuclear receptor subfamily 0, group B, member 1)	Nuclear receptor	Overexpression of *Dax1* (*Dax:Dax* transgene) in XY mice leads to impaired testis cord formation and spermatogenesis; the severity of the phenotype is dependent on the genetic background from which *Sry* originates Delayed testis development on CBA × C57BL/10 background, and hermaphrodites or female mice on C57BL/6 XY^{POS} XY background; conclusion: *Dax1* is an anti-testis gene[98] In *Nr0b1*$^{-/Y}$ (*SRY*^{POS} on 129SvJ) background, XY^{POS} mice show male-to-female sex reversal; conclusion: *Dax1* is a pro-testis gene[99]	Swain *et al.* (1998)[40] Meeks *et al.* (2003)[39]
Six1–Six4 (Six homeobox 1; (Six homeobox 4)	Transcription factor	*Six1*$^{-/-}$–*Six4*$^{-/-}$ XY mice have male-to-female sex reversal and/or ovarian development (in the majority of XY animals) No gonadal phenotype in *Six1*$^{-/-}$ mice or *Six4*$^{-/-}$ mice	Fujimoto *et al.* (2013)[60]
Sox3 (Sry-box 3)	Transcription factor	Knockout mice have no sex-determination phenotype, but are deficient in gonadal function (XX and XY)[100] XY mice with a transgene leading to overexpression of Sox3 in XY gonads have male-to-female sex reversal	Weiss *et al.* (2003)[100] Sutton *et al.* (2011)[73]
Sox8 (Sry-box 8)	Transcription factor	*Sox8*$^{-/-}$ mice have reduced fertility *Sox8*;*Sox9* double knockout XY mice have variable degree of male-to-female sex reversal	Sock *et al.* (2001)[45] Chaboissier *et al.* (2004)[27]
Sox9 (Sry-box 9)	Transcription factor	*Sox9*$^{-/-}$ XY mice have male-to-female sex reversal[28] *Ods* (deletion upstream of *Sox9*) XX mice have female-to-male sex reversal Ectopic expression in XX mice leads to female-to-male sex reversal	Barrionuevo *et al.* (2006)[28] Bishop *et al.* (2000)[29] Vidal *et al.* (2001)[30]
Sox10 (Sry-box 10)	Transcription factor	No gonadal phenotype *Sox10* overexpression leads to female-to-male sex reversal	Polanco *et al.* (2010)[47]
Sry (sex determining region Y)	Transcription factor	*Sry*$^{-/-}$ XY mice have male-to-female sex reversal Sry translocation in XX mice leads to female-to-male sex reversal	Koopman *et al.* (1991)[21]
Zfpm2, also known as *Fog2* (zinc finger protein, FOG family member 2)	Cofactor of Gata4	*Fog2*$^{-/-}$ mice have reduced Sry levels; XY mice have male-to female sex reversal	Bouma *et al.* (2007)[101]

Abbreviation: E, embryonic day.

The precise roles of every transcription factor in the gonadal network are not as well understood as those of *Sox9* and *Sry*. For example, *Nr0b1* (also known as *Dax1*) is considered both a pro-testis[39] and an anti-testis gene.[40] C57BL/6JEi mice with a mutant *Dax1* allele showed complete male-to-female sex reversal.[41] Although these mice expressed normal levels of *Sry* at the correct developmental stage, *Sox9* expression in these animals was impaired and Sertoli cell differentiation was not initiated, which resulted in failure of testis development. Normal testis development was recovered in these mice by overexpression of *Sry* in the XY gonads.[41] These findings suggest that upregulation of *Sox9*, which leads to Sertoli cell differentiation in XY gonads, is dependent on precisely timed expression of *Sry*, and *Dax1;* however, the exact molecular mechanism that underlies the rescue of the sex reversed phenotype by *Sry* is unknown. One possible explanation is that the high levels of *Sry* override *Dax1* deficiency and drive upregulation of *Sox9*. By contrast, in humans, inactivation or loss of *NR0B1* results in X-linked congenital adrenal hypoplasia,[42] whereas duplications of *NR0B1* lead to 46,XY gonadal dysgenesis.[43] To accommodate these apparently opposite roles, it has been suggested that *NR0B1* acts within a narrow window[44] and that the activity and concentration of NR0B1 is critical—too little or too much can lead to development of dysgenic testes. The contribution of *Dax1* to testis development in mammals is complex and its precise function remains unclear.

Together, *Sox8*, *Sox9* and *Sox10* comprise a single group of genes that share a higher degree of homology to each other than to other *Sox* genes. *Sox8* is expressed in the mouse testis cords at E13.5 and can induce *Amh* expression *in vitro*. Although *Sox8*-null XY mice seem to have normal testicular-like structures, they have dysfunctional adult Sertoli cells and reduced fertility.[45,46] Unlike mice that are null for either *Sox9* or *Sox8* alone, *Sox9;Sox8* double knockout mice show complete absence of testis cord formation,[27] a finding that supports functional redundancy between Sox9 and Sox8 in testis differentiation. Given that all *Sox* genes contain the highly conserved HMG-box that mediates DNA binding, this domain is likely to account for the proposed redundancy. Another explanation for the sex-reversal observed in *Sox9;Sox8* double knockout mice is that testis formation requires the expression of a threshold level of HMG-box proteins. By contrast, *Sox10*-null mice do not exhibit a testis phenotype; however, overexpression of *Sox10* in XX gonads results in development of testes, which suggest that this gene is sufficient, but not required, for testis differentiation.[47]

Key regulators of *Sry* expression

Since the discovery of *Sry* in 1990,[19,21,22] researchers have directed their attention to understanding the mechanisms that regulate its expression. Over the past two decades, several genes, including *Gata4, Zfpm2* (also known as *Fog2*),[48] *Wt1*[13,49] and *Nr5a1*[50–52] have been identified as positive regulators of *Sry* expression. *Cbx2* is also potentially upstream of *Sry* in the testis-determining pathway, as analysis of the expression profiles of *Cbx2*-null mice shows that this gene is required for the upregulation of all known positive regulators of *Sry*, in addition to *Sry* itself and downstream targets of Sry, such as *Sox9*.[15] Additionally, *Cbx2* has been implicated in a pathway downstream of *Sry* that regulates gonad size.[15]

Gata4 and Zfpm2

Gata4 is expressed early in the bipotential gonad; however, its expression becomes sexually dimorphic at E13.5, with both the gene and protein being expressed at high levels in developing Sertoli cells. *Gata4*-null mice die between E7.0 and E9.5 owing to severe abnormalities of heart-tube formation,[53,54] which makes analysis of gonadal formation in these mice challenging. Similarly, *Zfpm2*-null mice die from complications associated with heart defects, although these mice survive until E14.5, which enables analysis of the developing gonads.[48,53,55] Sex reversal was observed in both *Zfpm2*-null mice and mice homozygous for a targeted mutation in *Gata4* (*Gata4*^{ki}) that ablates the interaction between Gata4 and Zfpm2. The expression level of *Sry* in gonads isolated from *Zfpm2*-null mice at E11.5 was 25% of that detected in wild-type gonads.[56] Expression of genes that are downstream of *Sry* and are required for either Sertoli cell differentiation (such as *Sox9*, *Amh* and *Dhh*) or Leydig cell function (including *Cyp11a1*, *Hsd3b1* and *Cyp17a1*) was abolished in the gonads of *Zfpm2*-null mice.[41] As shown by use of the *Gata4*^{ki} mice, Gata4–Zfpm2 dimers are required for Sry regulation,[56] as well as for ovary development;[56] however, both proteins also seem to have separate and distinct functions during gonad development.[57] Gata4 on its own seems to be required for testis differentiation, testis cord formation and the transcriptional regulation of *Dmrt1*; furthermore, its temporal expression seems to be crucial for testis differentiation.[57] Gonadal loss of *Zfpm2* leads to an early, partial block of testis differentiation and consequently to male-to-female sex reversal.[57]

Expression of *Sry* is also regulated by the transcription factors Six1 and Six4;[58] these proteins, which are homologues of the *Drosophila sine oculis* homeobox (Six) family of DNA-binding proteins, contain Six domains and homeobox domains that display individual DNA-binding abilities.[58,59] Six1 and Six4 have similar expression profiles during embryogenesis.[58] Mice that are null for either *Six1* or *Six4* undergo seemingly normal gonadal development, which suggests that these proteins might be functionally redundant. However, in *Six1;Six4* double knockout mice, the gonads of the majority of XY embryos showed features of ovarian development with a concomitant reduction in *Sry* expression.[60] The gonads of both XX and XY offspring were reduced in size; and a detailed histological analysis showed reduced numbers of gonadal precursor cells at the time of initiation of *Sry* expression. Expression levels of known regulators of *Sry* transcription, such as *Zfpm2*, were also decreased. Conversely, overexpression of *Sry* rescued the impaired testis differentiation phenotype, but was unable to correct the reduced size of the gonads. Results of subsequent functional analyses indicated that Six1 and

Six4 function to directly activate *Zfpm2,* which in turn regulates expression of *Sry*.[60]

Another target of Six1 and Six4 that was identified in this study was *Nr5a1*.[60] In *Six1;Six4* double knockout mice, reduced numbers of Nr5a1-positive gonadal precursor cells were detected in the developing genital ridge at E9.5 and E10.5. *In vitro* experiments showed that Six1 and Six4 bind upstream of the *Nr5a1* gene and activate its expression. Importantly, *Nr5a1* expression was restored in the gonads of *Six1;Six4* double knockout mice by E13.5, which indicates that Six1 and Six4 do not regulate *Nr5a1* expression in differentiated gonadal somatic cell lineages. Thus, the *Six1–Six4–Zfpm2* axis seems to be required for precise spatiotemporal regulation of *Sry* expression and increased expression of *Nr5a1* mediated by Six1 and Six4 seems to control formation of gonad precursor cells and determine gonad size.

The mitogen-activated protein kinase pathway

One of the first indications that the mitogen-activated protein kinase (MAPK) pathway might be involved in testis formation came from a forward-genetic screen in mice that identified the boygirl (*byg*) variant in the *Map3k4* gene. XY animals homozygous for this variant on a C57BL/6J background showed male-to-female sex reversal, which was associated with a considerable decrease in levels of *Sry* expression.[61] The dramatic reduction in *Sry* mRNA and Sry protein levels accounts for the absence of Sertoli cells and subsequent formation of ovaries in these XY mice. Given that MAPKs are ubiquitously expressed, it is unclear how a null mutation in *Map3k4* would exert such a specific effect on *Sry* expression and testis formation; however, some evidence suggests that Map3k4 interacts with other cofactors, such as growth arrest and DNA damage-inducible protein γ (encoded by *Gadd45g*), which determine the spatiotemporal specificity of *Map3k4* in the testes.[62–64]

In the somatic cell lineages of both sexes, *Gadd45g* is expressed between E10.5 and E13.5, which overlaps with the temporal expression profile of *Sry*. On C57BL/6J[64] and C57BL/N[63] backgrounds (but not on a mixed genetic background),[65] *Gadd45g*-deficient XY mice present with male-to-female sex reversal. On either of the C57BL backgrounds, *Sry* expression is delayed in the absence of *Gadd45g*, which results in substantially lower levels of *Sry* mRNA and Sry protein at E11.5 than would be expected at this stage of development. The Sry protein levels observed in *Gadd45g*-deficient XY mice were similar to those in *Map3k4*-null mice. Additionally, both *Map3k4*-null and *Gadd45g*-null mice show reduced levels of phosphorylated (activated) p38 Mapk in embryonic gonads (at E9.5–E10, a time frame that corresponds to tail somite stages 15 and 16). Furthermore, disruption of p38 Mapk signalling, either chemically *ex vivo*[64] or by conditional gene targeting of p38α and p38β MAPK isoforms *in vivo,*[63] resulted in disrupted testis formation. The *in vivo* studies also showed reduced levels of *Sry* expression, as well as reduced levels of phosphorylated Gata4, which is a known target of MAPK signalling. Together, these findings support a role of the Gadd45g–Map3k4–p38 pathway in phosphorylation and activation of Gata4, in addition to spatiotemporal regulation of *Sry* transcription.

Insulin receptor tyrosine kinases

Insulin receptor tyrosine kinases also have a role in early testis formation. Triple knockout XY mice deficient for insulin receptor (*Insr*), insulin-like growth factor type 1 receptor (*Igf1r*) and insulin receptor-related protein (*Insrr*) showed complete male-to-female sex reversal and reduced expression levels of *Sry* and *Sox9*.[66] This phenotype suggests that these receptor tyrosine kinases are required for correct regulation of *Sry.* Other studies have demonstrated that loss of just two tyrosine kinases, *Insr* and *Igf1r*, is sufficient to cause the XY sex reversal phenotype.[67] Moreover, in addition to delayed *Sry* expression at E11.5, these double knockout animals demonstrate a reduction in Nr5a1-positive cells and delayed ovary development in both the XX and the XY (sex-reversed) gonads until E16.5.

Epigenetic regulation of Sry

Epigenetics is also involved in the regulation of *Sry* expression. Several histone modifications are associated with the different states of chromatin packaging that modulate the level of gene transcription, among them the methylation status of lysine residues 9 and 4 of histone H3 (H3K9 and H3K4, respectively). Methylated H3K9 is a marker of suppressed gene expression, whereas methylated H3K4 is a marker of activated gene expression. Lysine-specific demethylase 3A (encoded by *Kdm3a* [*Jmjd1a*]) is an enzyme that demethylates H3K9 residues and is, therefore, required for the activation of gene expression. *Jmjd1a*-deficient mice showed varying degrees of XY sex reversal, with the frequencies of complete ovarian development, one ovary and one testis or normal testis development dependent on the genetic origin of the Y chromosome (CBA or B6).[3] Detailed analysis of the gonads of *Jmjd1a*-deficient XY mice revealed morphological features of ovotestes at E13.5 (such as presence of both Sox9-positive and Foxl2-positive cells), a finding that is indicative of an early failure in the testis-determining pathway.[3] In wild-type mice, *Jmjd1a* mRNA levels increased in the somatic cells of the gonad from E10.5 to E11.5, and in *Jmjd1a*-deficient mice levels of H3K9me2 (twice methylated lysine 9) increased by approximately two-fold. By contrast, at E11.5, *Jmjd1a*-deficient mouse gonads exhibited reduced levels of *Sry* and *Sox9* transcripts; however, the levels of other known regulators of *Sry* were unaffected. *Jmjd1a* deficiency resulted in an increase of H3K9me2 levels without changes in H3K9me3 levels in *Sry* regulatory regions, which was indicative of H3K9me2-specific demethylation by Jmjd1a. H3K9me2 levels within the *Sox9* locus were unchanged, which excluded the possibility that Jmjd1a is a direct regulator of *Sox9* expression. Thus, Jmjd1a might regulate *Sry* expression via H3K9 demethylation, which drives changes in the chromatin structure of the *Sry* locus that permit transcription factors to bind *Sry* regulatory elements to induce *Sry* expression.

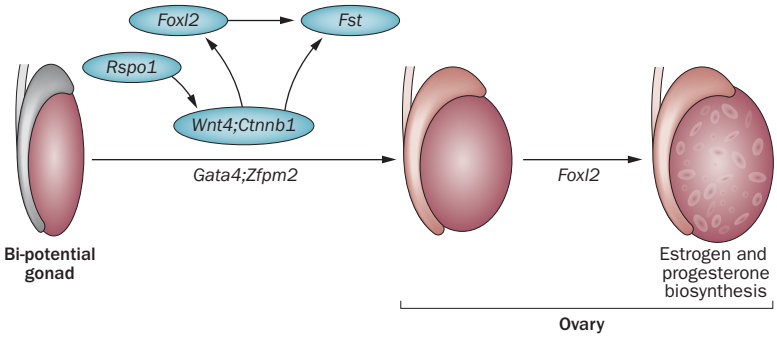

Figure 3 | Genes and pathways required for ovary development and differentiation. In XX mice, genes such as *Rspo1*, *Wnt4* and *Foxl2* are expressed during ovary development. *Rspo1*$^{-/-}$ ovaries show reduced levels of *Wnt4* expression, suggesting that Rspo1 acts upstream of *Wnt4*. However, a synergistic action of Wnt4 and Rspo1 in activating β-catenin (*Ctnnb1*) has also been suggested. Together, Foxl2, Rspo1 and Wnt4 activate *Fst* expression. In addition to these genes, the Gata4–Zfpm2 (also known as Fog2) pathway has been implicated in embryonic ovary formation. In the adult ovary, Foxl2 is required for tissue maintenance and follicle maturation.

Table 3 | Genes involved in regulation of ovary development

Gene (protein)	Protein function	Gonad phenotype in mouse models	Reference
Ctnnb1, also known as β-catenin (catenin, β1)	Transcription factor	Ectopic expression of the stabilized β-catenin gene causes male-to-female sex reversal	Maatouk *et al.* (2008)[86]
Foxl2 (forkhead box L2)	Transcription factor	Postnatal conditional (inducible) knockout causes transdifferentiation of ovarian somatic cell lineages (theca and granulosa cells) into testicular Sertoli and Leydig cells[82] Loss-of-function leads to female-to-male sex reversal in goats[81]	Boulanger *et al.* (2014)[83] Uhlenhaut *et al.* (2009)[84]
Rspo1 (R-spondin 1)	Signalling molecule	XX mice have partial female-to-male sex reversal[102]	Tomizuka *et al.* (2008)[102]
Wnt4 (wingless-type MMTV integration site family, member 4)	Signalling molecule	XX mice have Müllerian duct agenesis, testosterone synthesis and coelomic vessel formation	Vainio *et al.* (2004)[103]

Ectopic gene expression in the gonads

Given the high degree of conservation of the HMG-box region among *Sox* genes, genes with functions that are normally unrelated to gonad development, such as *Sox3*, could (under certain conditions) possibly mimic the function of *Sry*. The sex chromosomes are thought to have evolved as an autosomal pair and the Y chromosome is proposed to be simply an X chromosome that has degenerated over time.[68,69] Thus, some genes on the X chromosome have survived on the Y chromosome; however, their function has changed to fulfil male-specific roles.[70,71] Examples of this neofunctionalization are the X-linked *Sox3* and Y-linked *Sry* genes. These genes are structurally identical, but functionally distinct. Mutations in human *SOX3* have been implicated in development of X-linked intellectual disability,[72] whereas *SRY* is the key Y-linked gene that initiates testis formation. *Sox3* is not expressed in the developing gonads of either sex in mice; however, a transgenic mouse line in which *Sox3* was ectopically overexpressed in the bipotential gonad showed XX female-to-male sex reversal.[73] *In vitro* cotransfection experiments demonstrated that *Sox3* can functionally replace *Sry* and induce *Sox9* expression via synergistic action with Nr5a1.[73]

Hedgehog signalling

Hedgehog signalling is an important pathway in embryonic development that controls patterning and differentiation of a variety of different organs.[74] Desert hedgehog (*Dhh*) is the sole member of the hedgehog signalling family that is expressed in the developing mouse gonad. Expression of *Dhh* in XY gonads begins at E11.5 and continues during differentiation of the Sertoli cells; however, *Dhh* expression has not been detected in XX gonads at any developmental stage.[75–77] Differentiated Sertoli cells synthesize and secrete Dhh, which subsequently binds to its receptor, protein patched homologue 1 (Ptch1) on Leydig and peritubular myoid cells. Mice that are null for *Dhh* display a disrupted testis cord phenotype that is consequent to development of abnormal peritubular tissue.[78,79] Dhh signalling is also required for upregulation of *Nr5a1* and *Ptch1* expression in Leydig cells.[75,76]

An additional gene involved in hedgehog signalling that probably contributes to gonadal development is hedgehog acyl-transferase (*HHAT*), which was initially identified in a patient with a homozygous variant in this gene that resulted in 46,XY testicular dysgenesis. HHAT mediates palmitoylation of hedgehog signalling proteins. At E12.5, embryos of mice that lack *Hhat* have reduced testis size and a dysgenic testis cord phenotype.[80]

Ovary determination

Much less is known about the regulation of ovary development than testis development. However, a number of genes have been identified that together provide increased understanding of the previously enigmatic developmental regulation of this organ (Figure 3; Table 3).

Foxl2

Forkhead box L2 (*Foxl2*) is a member of the forkhead box gene family, which encodes an evolutionary conserved set of transcription factors. In the mouse, *Foxl2* is one of the earliest genes to be upregulated in the developing female-specific ovary, suggesting an important function for this gene in early ovary differentiation. Overexpression of *Foxl2* in XY mice and ablation of *Foxl2* expression in XX mice results in gonadal anomalies, but not sex reversal.[81] In goats, however, *FOXL2*-null mutations or deletion of a 11.7 kb region upstream of *FOXL2* lead to female-to-male sex reversal,[82,83] which further supports a role for this gene in female-specific development. Interestingly, in mice at least, Foxl2 also has a role in postnatal maintenance of the ovary, where it functions to suppress genes involved in testis differentiation from early embryonic gonad differentiation throughout adult life.[84]

Wnt4, Rspo1 and β-catenin

Two components of the Wnt signalling pathway, Wnt4 and Rspo1, have crucial roles in ovarian development.

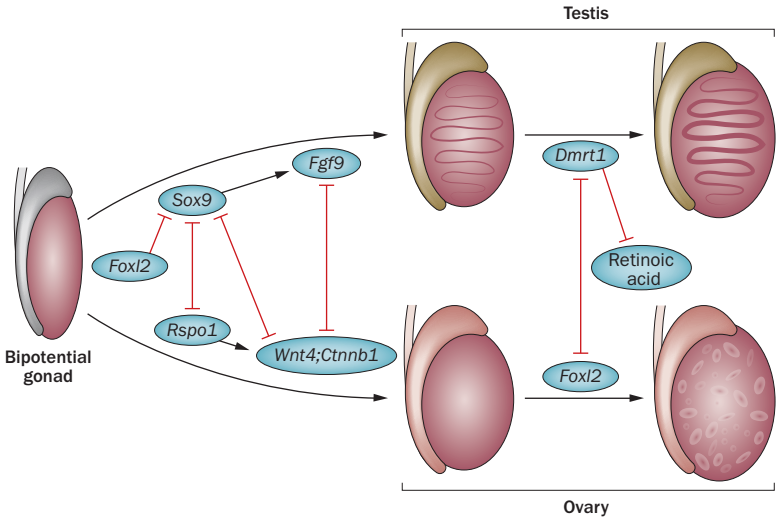

Figure 4 | Interactions between the testis-specific and ovary-specific pathways during embryonic development and adulthood. Multiple genes and proteins interact between the two pathways, ensuring that once one developmental pathway is activated, the other pathway is repressed. During embryonic gonad development, the ovary-specific genes *Foxl2*, *Rspo1*, *Wnt4* and *Ctnnb1* (β-catenin) suppress the male pathway through inhibition of *Sox9* and *Fgf9* expression. Sox9 and Fgf9 can inhibit the ovarian pathway by suppressing *Rspo1* and *Wnt4–Ctnnb1* expression, respectively. Even during adult life, the structures of the testis and ovary need to be maintained by actively suppressing the pathway of the opposite sex. Dmrt1 has a role in the maintenance of adult Sertoli and Leydig cell populations through inhibition of the ovarian retinoic acid and Foxl2 pathways, which would otherwise lead to the development of ovarian granulosa and theca cells. By contrast, Foxl2 is required in the adult ovary to suppress *Dmrt1* expression and prevent transdifferentiation of granulosa and theca cells into testicular Sertoli and Leydig cells.

Wnt4 and *Rspo1* function via activation of β-catenin (Ctnnb1 protein), which in turn regulates transcription of a variety of genes, among them important ovarian components, such as *Wnt4 and Fst*.[85,86] Expression of a stabilized form of β-catenin in somatic cells of the developing mouse XY gonad is sufficient to cause male-to-female sex-reversal.[86] However, further studies will be required to elucidate the exact mechanisms that drive sex reversal in these animals as well as the precise role of stabilized β-catenin in activation of ovary-specific genes and/or repression of testis-specific genes.

Interplay of testis and ovary pathways

Female development has traditionally been considered the default pathway in mammalian species with XY–XX genetic sex determination. The basis of this assumption is that if the gonads are surgically removed before sex determination, or if they fail to develop owing to loss-of-function mutations in crucial early-gonad genes, a genetically male embryo will develop as a phenotypic female.[87,88] However, emerging data suggest this is an overly simplistic view and that complex interactions exist between testis and ovary developmental pathways (Figure 4).

Shortly after the discovery of *SRY* as the key testis determinant, the 'Z model' hypothesis was proposed.[89] Under this hypothesis, a so-called 'Z' factor was produced by the XX gonad to actively promote ovary development by suppressing one or more pro-testis genes. According to this model, *Sry* or another early male-specific gene functions to suppress ovarian development by blocking the activity of the Z factor. Although no Z factor has yet been identified, several studies provide strong evidence that male and female pathways actively suppress each other during gonad differentiation and after functional testes or ovaries have fully developed.[84,90,91]

Given the fundamental role of *Sox9* in early testis development, it is not surprising that regulation of its expression and activity is central to the interactions of the pathways that specify formation of either the testis or the ovary. In the mouse, Sry and Nr5a1 have been shown to up-regulate *Sox9* via the TESCO enhancer.[10] After expression of *Sry* ceases, Sox9 maintains its own expression via this testis-specific enhancer, thereby allowing testis differentiation to proceed. *In vitro* experiments have shown that the female-specific transcription factor Foxl2 can bind to TESCO and suppress enhancer activity, thereby preventing high levels of *Sox9* expression in the developing ovary.[84] TESCO might act synergistically with other regulatory elements, potentially through three-dimensional looping, leading to initiation, upregulation and self-maintenance of *Sox9* expression.[34]

An additional target of Foxl2 is *Nr5a1*. By antagonizing Wt1 –KTS, Foxl2 represses *Nr5a1* expression in the developing ovary.[92] Two other major opposing components of gonadal differentiation are the signalling molecules Fgf9 and Wnt4. In testis development, Fgf9 is strongly upregulated and functions to maintain *Sox9* expression, as well as to downregulate expression of female-specific genes such as *Wnt4*. Sox9 in turn activates the expression of *Fgf9*.[93] In the ovary, this feedback loop is blocked by Wnt4, possibly via the activation of β-catenin, which either directly or indirectly suppresses the upregulation of *Sox9*.[94] Fgf9 downregulates expression of *Wnt4* in the developing testis and prevents disruption of the Sox9–Fgf9 loop.[95]

Molecular mechanisms are present in the postnatal gonads of both sexes that maintain the differentiated ovarian or testicular structure and prevent activation of the opposing developmental pathway. Conditional deletion of *Foxl2* from mature granulosa cells of female mice resulted in development of testis-like features in the ovaries, including transdifferentiation of granulosa and theca cells into Sertoli and Leydig cells, respectively. The gonads of these mice resembled testes, with seminiferous tubules instead of the typical ovarian follicular structure.[84] These experiments clearly show that Foxl2 is required to suppress the testicular pathway and maintain the ovaries, both during embryonic development and throughout adult life.

In mature XY gonads, Dmrt1 maintains the testis pathway and suppresses the ovarian pathway. Knockout of *Dmrt1* in Sertoli cells of postnatal XY mice induces transdifferentiation of these cells into a granulosa-like phenotype: germ cells become feminized and the gonads start to produce estrogen. Interestingly, *Foxl2* expression is also induced, which might account for the activation of the female pathway.[90] A second study showed that *Dmrt1* and *Sox9* are required for the maintenance of the postnatal testis via suppression of female sex-determining genes, such as *Foxl2*.[91] Dmrt1 also seems to be required for the

suppression of excessive retinoic acid signalling in the testis, which acts to further block the expression of female sex-determining genes. Taken together, the findings from these studies show that both testes and ovaries are surprisingly labile (even after birth), which indicates that constitutive expression of genes is required to actively suppress the opposing pathway and prevent transdifferentiation.

Regulatory RNA species

Protein-coding genes have been the focus of the majority of research on sex differentiation and gonad development to date. However, development of new technologies (such as single-nucleotide-polymorphism arrays and deep sequencing) has made analysis of the noncoding regions of the genome feasible. Another emerging area of research addresses the function and involvement of noncoding RNA species (such as microRNAs, long noncoding RNAs and PIWI-interacting RNAs). Although the amount of published data is still very limited, a few studies have investigated the involvement of microRNAs in sex differentiation. In developing mouse ovarian cells, miR124 downregulates expression of *Sox9*,[96] whereas in the developing testis, Sox9 drives increased expression of miR202-5p and miR202-3p.[2] Although the precise functions of miR202-5p and miR202-3p remain to be determined, they are proposed to be involved in early testis differentiation.[2] Additionally, miR140-3p and miR140-5p have been implicated in Leydig cell differentiation.[97] These findings might provide insights into the importance of post-transcriptional sex-specific regulation of autosomal genes and other (yet to be identified) noncoding RNAs are also very likely to be involved in sex differentiation.

Conclusions

Over the past few decades, our understanding of the regulation of gonad specification and subsequent sex differentiation has increased with the identification of new genes that modulate *Sry* expression. In addition, new layers of gene regulation underpinning gonad development (such as epigenetic modification and regulatory RNA species) have been uncovered. Nevertheless, despite this increased understanding, a comprehensive picture of the regulatory networks required for a fully functional testis or ovary is still lacking. Discoveries such as the identification of *Jmjd1a* and its epigenetic regulation of testis development clearly demonstrate that all levels of gene regulation must be examined if we are to fully understand the complex network that regulates gonad differentiation.

Review criteria

A PubMed search for full-text papers in English published up to July 2014 was performed using the search terms "male-to-female sex-reversal", "female-to-male sex-reversal", "sex determination", "gonad development", "testis development", "ovary development". Bibliographies of manuscripts included in this Review were also used to identify further relevant articles.

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Acknowledgements
The authors' research work was supported by The National Health and Medical Research Council, Australia (program grant #546517 and project grant #1031214); the Helen Macpherson Smith Trust (partnership grant #6846); the Ian Potter Centre for Genomics and Personalised Medicine; and the Victorian Government's Operational Infrastructure Support Program.

Author contributions
All authors contributed equally to all aspects of this manuscript.