Molecular mechanisms involved in mammalian primary sex determination

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Abstract

Sex determination refers to the developmental decision that directs the bipotential genital ridge to develop as a testis or an ovary. Genetic studies on mice and humans have led to crucial advances in understanding the molecular fundamentals of sex determination and the mutually antagonistic signaling pathway. In this review, we summarize the current molecular mechanisms of sex determination by focusing on the known critical sex determining genes and their related signaling pathways in mammalian vertebrates from mice to humans. We also discuss the underlying delicate balance between testis and ovary sex determination pathways, concentrating on the antagonisms between major sex determining genes.

Key Words

- ▶ sex determination
- ▶ sexual differentiation
- ▶ Sertoli cell
- ▶ genital ridge

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Introduction

How an individual's sex is determined and coordinated has been a long-standing question in embryology and developmental biology. In the animal kingdom, the genetic and molecular mechanisms involved in sex determination vary remarkably. Over the past couple of decades, studies of sex determination and sexual differentiation have revealed that sex in vertebrates is regulated by a broad variety of mechanisms based on genotypes or environmental signals.

Sex development can be divided into two categories, namely sex determination and sexual differentiation. Sex determination is defined as the developmental decision that directs the bipotential gonad to develop as a testis or an ovary. In mammals, sex determination is genetically controlled depending on a developmental time and gene expression.

Since the discovery of the sex determining region of chromosome Y (*SRY*) in 1990 (Berta *et al.* 1990, Gubbay

et al. 1990, Sinclair *et al.* 1990), research efforts have led to the identification of *SRY* as the master gene in the genetic cascade required for sex development in most mammals. Meanwhile, many crucial components and events have been documented at the molecular and cellular level in humans and mice. Compelling evidence indicates that mammalian sex-determining cascades reveal some general features.

In this review, we summarize the molecular mechanisms of sex determination and sexual differentiation by focusing on general molecules and the conserved pathways in mice and humans. Specifically, we describe the current understanding on mutual cross-talks between central molecules in sex development (Table 1), such as male sex-determining genes *FGF9*, *SOX9*, *DMRT1* and female sex-determining genes *DAX1* (*NROB1*), *FOXL2*, *RSPO1*, and *WNT4*, and highlight new insights into the delicate balance between the testis- and ovary-determining pathways.

Table 1 Genes involved in the male and female sex determination pathway

Gene	Protein function	Gain- and loss-of-function phenotypes		References
		Human syndrome	Mouse models	
Genes involved in initial development				
<i>Emx2</i>	Transcription factor	–	Aberrant tight junction assembly, failure in genital ridge formation (LOF)	Kusaka et al. (2010)
<i>Gata4</i>	Transcription factor	Ambiguous external genitalia, congenital heart disease (LOF)	Failure in thickening of the coelomic epithelium, defective initial formation of genital ridge (LOF)	Lourenço et al. (2011), Manuylov et al. (2011) and Hu et al. (2013)
<i>Wt1</i>	Transcription factor	Denys-Drash, Frasier syndrome (LOF)	Disruption of seminiferous tubule and somatic cell apoptosis, XY sex reversal (LOF)	Kreidberg et al. (1993), Hammes et al. (2001) and Gao et al. (2006)
<i>Lhx9</i>	Transcription factor	–	Failure in genital ridge formation (LOF)	Birk et al. (2000)
<i>Sf1</i>	Nuclear receptor	Embryonic testicular regression syndrome, gonadal dysgenesis	Delayed organization of male testis cord, failure in genital ridge formation (LOF)	Park et al. (2005) and Lin et al. (2007)
Genes involved in the regulation of SRY expression during primary sex determination				
<i>Gata4/Fog2</i>	Transcription/cofactor	–	Apparent XY gonadal sex reversal (LOF)	Tevosian et al. (2002)
<i>Gadd45γ</i>	Stress-response protein	–	XY sex reversal (LOF)	Gierl et al. (2012) and Warr et al. (2012)
<i>Map3k4</i>	Kinase	–	XY sex reversal (LOF)	Bogani et al. (2009)
<i>Cbx2</i>	Transcription factor	XY ovarian DSD, XY sex reversal (LOF)	XY sex reversal (LOF)	Blason-Lauber et al. (2009) and Katoh-Fukui et al. (2012)
<i>Irf1, Irf1r</i>	Membrane receptor	–	XY sex reversal (LOF)	Nef et al. (2003)
Genes involved in male testis determination pathway				
<i>SRY</i>	Transcription factor	Turner syndrome, Klinefelter syndrome, XY sex reversal (LOF)	XY sex reversal (LOF); XX sex reversal (GOF)	Ford et al. (1959), Jacobs & Strong (1959), Koopman et al. (1989) and Gubbay et al. (1990)
<i>Sox9</i>	Transcription factor	Campomelic dysplasia XY sex reversal (LOF)	Abnormal Sertoli cell differentiation, XY sex reversal (LOF); XX sex reversal (GOF)	Foster et al. (1994), Huang et al. (1999), Vidal et al. (2001), Chaboissier et al. (2004) and Barrionuevo et al. (2006)
<i>Amh</i>	Hormone	XY sex reversal (LOF)	XY sex reversal (LOF)	Kim et al. (2006) and Jameson et al. (2012)
<i>Fgf9</i>	Growth factor	XY sex reversal (LOF)	XY sex reversal (LOF)	Matson et al. (2012)
<i>Dmrt1</i>	Transcription factor	XY gonadal dysgenesis, XY sex reversal (LOF)	Postnatal feminization in XY mice, defective seminiferous tubule (LOF)	

Table 1 Continued

Gene	Protein function	Gain- and loss-of-function phenotypes		References
		Human syndrome	Mouse models	
Genes involved in female ovary determination pathway <i>Wnt4</i>	Signaling molecule	Ambiguous genitalia (GOF)	Failure in the formation of coelomic vessel and germ cell, degeneration of the female reproductive tract, partial XX sex reversal (LOF)	Jordan <i>et al.</i> (2003) and Yao <i>et al.</i> (2004)
β -catenin <i>Rspo1</i>	Transcription factor Growth factor	–	Partial XX sex reversal (LOF)	Chassot <i>et al.</i> (2008)
<i>Foxl2</i>	Transcription factor	Palmoplantar hyperkeratosis, skin carcinoma, complete XX sex reversal (LOF) BPES (LOF)	Development of ovotestes, partial XX sex reversal (LOF)	Parma <i>et al.</i> (2006) and Chassot <i>et al.</i> (2008)
<i>Dax1</i>	Nuclear receptor	XX sex reversal (LOF)	Premature ovarian failure, ablation of the primordial follicle pool, partial XX sex reversal (LOF) XX sex reversal (LOF)	Uhlenhaut & Treier (2006) and Ottolenghi <i>et al.</i> (2007) Swain <i>et al.</i> (1998) and Meeks <i>et al.</i> (2003)

–, no mutations have been identified to date in human sexual reversal patients; GOF, gain of function; LOF, loss of function; BPES, blepharophimosis/ptosis/epicanthus inversus syndrome.

Genes involved in the initial development of the bipotential gonad

During mammalian early fetal development, the indifferent, bipotential gonad first appears as symmetrically paired structures within the intermediate mesoderm. The bipotential gonad later can follow one of two alternative male or female fates and develop as two morphologically and functionally different tissues, a testis or an ovary (Kim & Capel 2006, Wilhelm *et al.* 2007a,b).

For normal growth and maintenance of the bipotential gonad a few genes are required, including *Drosophila* empty spiracles homeobox 2 (*EMX2*), GATA-binding protein 4 (*GATA4*), Wilms' tumor 1 (*WT1*), LIM homeobox 9 (*LHX9*), and steroidogenic factor 1 (*SF1*) (Table 1). These genes are now discovered to play definitive roles in the earliest gonadal development and primary sex determination (Brennan & Capel 2004, Sekido & Lovell-Badge 2013). These genes were shown to be essential for the normal growth and the maintenance of the coelomic epithelium that lies in the embryonic body cavity during genital ridge formation (Fig. 1A; Eggers & Sinclair 2012, Hu *et al.* 2013). Homozygous deletion of any one of these genes results in progressive degeneration of the emerging differentiated somatic cell lineages and disruption of the subtle structure of the genital ridge (Gao *et al.* 2006, Kusaka *et al.* 2010, Manuylov *et al.* 2011, Hu *et al.* 2013).

Drosophila empty spiracles homeobox 2

The paired-like homeobox gene *EMX2* homologous to the *Drosophila* empty spiracles (*ems*) gene shows similar expression pattern in the primordia of the urogenital system in both the male and female bipotential gonads (Pellegrini *et al.* 1997). In mice, *Emx2* is initially expressed in the pronephric primordium at E8.75–9.0 and then extends to the developing mesonephros and the coelomic epithelium by E10.0, finally *Emx2* is present in both mesospheric and paramesonephric ducts at E13.5 (Pellegrini *et al.* 1997). In *Emx2*-null mice, the urogenital system, including kidney, ureters, genital ridge, and genital tracts, is absolutely absent and the formation of the ureteric bud is disrupted (Miyamoto *et al.* 1997). Early stages of *Emx2*-knockout embryonic gonads display abnormal cell proliferation and disorganized migration of gonadal epithelial cells to the mesenchymal compartment (Kusaka *et al.* 2010). The aberrant tight-junction assembly of the gonadal epithelia cells and upregulated expression of *Egfr* in *Emx2*-KO embryonic gonads

Figure 1

Molecular crosstalk between critical genes in the initial development of the bipotential gonad. (A) Emerging molecular network involved in the initial formation and development of the bipotential genital ridge. *Emx2*, *Gata4*, *Wt1*, *Lhx9*, and *Sf1* are expressed in the molecular pathway regulating the early bipotential gonad development. GATA4 functions upstream of these genes to initiate the thickening of the coelomic epithelium, and together with *Lhx9*, regulate *Sf1* expression. WT1 (–KTS) and *Emx2* also upregulate the expression of *Sf1* in a developmental cascade. (B) *Emx2* represses EGFR signaling to coordinate the tight-junction assembly in the early developing gonad. *Emx2* inhibits the phosphorylation of c-Src and maintains the

implicate that *Emx2* is involved in the maintenance of the genital ridge epithelial cell polarity and the regulation of the epithelial-to-mesenchymal transition (Fig. 1B; Kusaka *et al.* 2010). The expression of *Sf1* and aristaless-related homeobox (*Arx*) is absent in the *Emx2*-KO gonads at E11.5, indicating that *Emx2* may directly or indirectly coordinate the expression of *Sf1* and *Arx* in the developing gonads, while the expression patterns of other genes, including *Gata4*, *Wt1*, and *Lhx9*, are intact in the *Emx2*-KO gonads (Kusaka *et al.* 2010). Recent studies on the *Pax2*^{+/-}; *Emx2*^{+/-} mice embryos have revealed that *Pax2* may directly regulate the *Emx2* expression in the Wolffian duct, and *Pax2* coordinates the ureter and kidney development (Boualia *et al.* 2011). The roles of *Emx2* in the developing genital ridge remain largely uncharacterized and the molecular mechanisms upstream and downstream of *Emx2* are poorly understood.

GATA-binding protein 4

GATA4, an evolutionarily conserved member of GATA transcription factors, is abundantly expressed in the developing supporting cell lines in the undifferentiated genital ridge of both male and female mouse embryos at E11.5 (Viger *et al.* 1998). Thereafter, *Gata4* presents an

normal *c-Src* in a low level in the normal epithelial cells. In the *Emx2*-KO gonad, the *c-Src* is phosphorylated at the tyrosine residue by EGFR and in turn phosphorylates EGFR at the tyrosine 845. (C) The GATA4-FOG2 complex is required for normal SRY expression and Sertoli cell differentiation. GATA4-FOG2 complex is also responsible for the expression of three major Sertoli cell-specific genes critical for normal Sertoli cell differentiation (*Sox9*, *Amh*, and *Dhh*) and three genes encoding androgen biosynthetic enzymes (*P450scc*, *3βHsd*, and *P450c17*). GATA4 inhibits the initial expression of *Wnt4*, the female sex-determining gene. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0018>.

obvious sexual dimorphic expression pattern, in which *Gata4* is dramatically downregulated in the female mouse embryo during ovarian differentiation and becomes restricted to Sertoli cells in different stages of testicular development (Viger *et al.* 1998).

Gata4 is initially expressed in the anterior part of the gonad at E10.0 and progresses in an anterior-to-posterior direction just before the initial thickening of the coelomic epithelium and the formation of the genital ridge (Tevosian *et al.* 2002, Hu *et al.* 2013). *Gata4* also functions in a precise time- and dosage-dependent manner, and the initial thickening of the coelomic epithelium and the growth of the genital ridge are dependent on *Gata4* rather than on other genes (Fig. 1C; Manuylov *et al.* 2011, Hu *et al.* 2013). The expression of *Lhx9* and *Sf1* in the coelomic epithelium is partially regulated by *Gata4*; *Lhx9* and *Sf1* function downstream of *Gata4* to support the initiation of genital ridge development (Hu *et al.* 2013). However, *Emx2* and *Wt1* act in parallel pathways independent of *Gata4* to promote the maintenance of the genital ridge epithelium (Hu *et al.* 2013).

Targeted deletion of *Gata4* in the mouse gonad leads to the failure of testis development and significantly decreased the expression of male-specific gene Doublesex and Mab3-related transcription factor-1 (*Dmrt1*) in Sertoli

cells (Manuylov *et al.* 2011). *In vitro* experiments also identified that GATA4 specifically recognizes three critical DNA response elements located within the distal regulatory region of the *Dmrt1* promoter, and GATA4 is required for robust expression of *Dmrt1* in the postnatal testis (Lei & Heckert 2004).

Wilms' tumor 1

Wt1 consists of ten exons and encodes a zinc finger transcription factor, which has at least 24 different isoforms due to alternative splicing, alternative translation start sites, and RNA editing (Hammes *et al.* 2001). Two alternative splice sites at the end of exon 9 results in the insertion or exclusion of the three amino acids (KTS; K-lysine; T-threonine; S-serine) between the third and fourth zinc fingers (designated as +KTS and -KTS isoforms) (Hammes *et al.* 2001).

The critical roles of WT1 in mammalian primary sex determination were discovered based on the phenotypes of genital tract dysgenesis and male-to-female sex reversal in human XY patients with germ line *Wt1*^{+/-} mutation (Pelletier *et al.* 1991), and this was also consistent with the apoptosis of the genital ridge and absolutely gonadal dysgenesis in the *Wt1*^{-/-} mice (Kreidberg *et al.* 1993). Conditional ablation of *Wt1* in Sertoli cells by E14.5 after primary testis determination results in the disruption of seminiferous tubule architecture and subsequent progressive loss of Sertoli cells and germ cells in mice (Gao *et al.* 2006). The complete absence of *Sf1* expression in the early genital ridge of *Wt1*^{-/-} mouse embryos suggests that WT1 is required for the normal expression of *Sf1* (Wilhelm & Englert 2002). The deletion of *Wt1* results in the progressive loss of *Sox8*, *Sox9*, and anti-Müllerian hormone (*Amlh*) in the mutant Sertoli cells at E14.5, after *Sry* expression is ceased, indicating WT1 is indispensable for the continued expression of these male-specific genes in a parallel way independent of SRY (Gao *et al.* 2006).

Taken together, WT1 is indispensable at multiple steps in mammalian sex development, including the early steps of sex determination and later stages of male testicular formation.

LIM homeobox 9

Lhx9, a member of the LIM homeobox domain gene family, is initially expressed in the medial surface of the urogenital ridge at E9.5; and later is restricted to the mesothelial layer and the interstitial region at E13.5 (Birk *et al.* 2000). In *Lhx9*^{-/-} mice, the gonad formation is

arrested, and the proliferation of the epithelium into the mesenchyme is absent in *Lhx9*^{-/-} embryos (Birk *et al.* 2000). *In vitro* biochemical experiments reveal that *Lhx9* and *Wt1* (-KTS) can directly bind to the *Sf1* promoter and upregulate the expression of *Sf1*. Analyses of *Lhx9*^{-/-}, *Sf1*^{-/-}, and *Wt1*^{-/-} mice suggest that *Lhx9* and WT1 (-KTS) cooperatively function upstream of *Sf1* in a developmental cascade and thus mediate the formation of the mammalian genital ridge and early gonadogenesis (Wilhelm & Englert 2002).

Steroidogenic factor 1

Sf1, encoded by the gene *Nr5a1* is an orphan nuclear receptor whose expression is first apparent in E9.0 in the adrenal/gonadal primordium (APG). *Sf1* persists in the bipotential gonad of both sexes, but exhibits a sexually dimorphic expression pattern in the developing gonads between E13.5 and E16.5 (Hoivik *et al.* 2010). In humans, heterozygous missense mutations in *Sf1* in 46,XY individuals can lead to primary adrenal failure, relatively severe gonadal dysgenesis, and impaired Müllerian structures (Lin *et al.* 2007). Meanwhile, *Sf1* knockout mice also exhibit failure of adrenal and gonadal development, and both male and female *Sf1*-deficient mice generate female internal genitalia (Luo *et al.* 1994). Cell-specific deletion of *Sf1* in the mouse gonads leads to the delayed organization of male testis cord and decreased somatic cells proliferation in the embryonic testis (Jeyasuria *et al.* 2003, Park *et al.* 2005). These studies indicate that SF1 is a crucial regulator in the development and differentiation of the mammalian reproductive system.

Master genes in the mammalian sex determination pathway

SRY is the master switch in mammalian sex determination

In 1959, reports on two human disorders of sex development (DSD), Turner syndrome (XO females) (Ford *et al.* 1959) and Klinefelter syndrome (XXY males) (Jacobs & Strong 1959), demonstrated the existence of a pivotal sex-determining gene on the human Y chromosome. It has been generally accepted that sex in mammals is determined by genes on the Y chromosome, called the testis-determining factor (*TDF* in humans or *Tdy* in mice) (Koopman *et al.* 1989, Mardon *et al.* 1989, Graves 2002). In 1990, a search of a 35-kb region of the human Y chromosome necessary for male sex determination resulted in the identification of the SRY as a candidate

for *TDF* (Berta *et al.* 1990, Sinclair *et al.* 1990). The cloning of the corresponding homologous sequences from the mouse Y chromosome led to the discovery of *Sry* to be the testis-determining gene in mice (Gubbay *et al.* 1990).

SRY is the founding member of the SOX (SRY-related high mobility group (HMG) box) family of transcription factors, which encode an evolutionarily conserved HMG DNA-binding domain. SRY comprises three different domains: N-terminal domain (NTD), HMG box, and C-terminal domain (CTD) (Fig. 2A). The HMG domain is highly conserved among species, and consists of a 79 amino-acid polypeptide. This domain comprises three α -helices that can form a L-signature shape and interacts with the consensus sequence (A/T)ACAA(T/A) in the minor groove of DNA with a significantly high affinity, and causes an approximate 65°–80° bend at the binding site (Giese *et al.* 1994, Chew *et al.* 2005, Palasingam *et al.* 2009, Jauch *et al.* 2012). Apart from the HMG domain, the regions outside the HMG domain of SRY are poorly conserved among mammals without any identified structural/functional domain. However, experimental evidence from the non-HMG-box domains, including the mutations in the NTD and CTD, lead to DSD in both transgenic mouse models and human DSD patients,

suggesting that the non-HMG-box domains may be involved in the transactivation of SOX9, and maintenance of an optimal protein structure, thereby contributing to the DNA-binding affinity and recruiting other crucial SRY partner proteins (Fig. 2B; Zhao & Koopman 2012).

Sry is the master gene that initiates male sex determination in almost all mammals except in the monotremes, such as the platypus and the echidna (Wallis *et al.* 2008). Gain-of-function study of the *Sry* indicates that a 14 kb mouse genomic DNA containing *Sry* is sufficient to induce testis differentiation and subsequent male development. It gives rise to completely XX male sex reversal into chromosomally female mouse embryos (Koopman *et al.* 1991). Evidence from a number of transgenic mouse models and human DSD patients also demonstrates that loss-of-function of *Sry* can lead to the XY gonadal dysgenesis or Turner syndrome with XO/XY mosaicism (Zhao & Koopman 2012).

Genes implicated in the regulation of SRY expression

Most of our current understandings of molecular and cellular mechanisms of mammalian sex determination come from studies on mice. In the mouse embryo, the

Figure 2

Schematic representations of functional domains of SRY protein and the upstream regulators of *Sry* during primary sex determination. (A) Schematic representation of the human and mouse SRY structure and functional domains. Both human and mouse SRYs are composed of three different domains: the N-terminal domain, the HMG box, the C-terminal domain. (B) Data acquired from the human and mouse SRYs are integrated into the model of the human SRY sequence. Human and mouse SRYs can be transported into the nucleus through two nuclear localization signals (NLSs) located in the N-terminus and C-terminus of HMG box. In the cytoplasm, calmodulin (CaM) can bind to the N-NLS, and β -importin (Imp β) can recognize the C-NLS and then translocate SRY into the nucleus.

The acetylation of the nuclear exportation site (NES) can enhance the exportation activity of SRY. (C) This figure indicates molecular cascades implicated in the regulation of SRY expression. WT1 (+KTS) coordinates the cell-autonomous modulation of *Sry* expression. The GATA4/FOG2 complex interacts with the *Sry* regulatory region to activate its expression. A p38 MAP3K4 signaling cascade, including GADD45 γ -p38 MAPK-GATA4-SRY, is involved in the regulation of normal SRY expression during sex determination. The insulin receptor tyrosine kinase family, comprising *Irr*, *Irrr*, *Igf1r*, and *Cbx2*, is responsible for the normal *Sry* expression during early stage of testicular differentiation. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0018>.

expression of *Sry* mRNA transcript initially begins in the somatic cells of XY genital ridges at E10.5, reaches a peak at E11.5 throughout the whole length of gonad, and wanes by E12.5 (Hacker *et al.* 1995, Bullejos & Koopman 2005). In mice, *Sry* is transiently expressed in a center-to-pole wave pattern along the anteroposterior axis of the bipotential XY gonads (Hiramatsu *et al.* 2010). Hiramatsu *et al.* (2009) established a heat shock-inducible *Sry* transgenic mouse system that allows precise experimental control over the timing onset of *Sry* expression, and they demonstrated that the ability of *Sry* to determine the testis fate is limited to approximately E11.0–11.25, a narrow and crucial time window for about only 6 h.

The precise regulation mechanisms of *Sry* expression in the bipotential gonad remain unknown as yet (Fig. 2C and Table 1). This is also reviewed by Sekido & Lovell-Badge (2013). WT1 (+KTS) is responsible for the high expression levels of *Sry* during formation of male gonad, and *in vitro* system WT1 (+KTS) can directly bind to the *Sry* promoter in order to transactivate *Sry* expression (Hammes *et al.* 2001, Hossain & Saunders 2001). In XY *Wt1* (+Kts)^{-/-} mouse gonads, a reduction in *Sry*-expressing cells numbers, correlating with ablation of male-specific coelomic epithelium proliferation, was observed, suggesting that WT1 (+KTS) coordinates the cell-autonomous modulation of *Sry* expression and Sertoli cell differentiation *in vivo* (Bradford *et al.* 2009).

GATA4 and its cofactor, friend of GATA2 (FOG2), are involved in gonadal differentiation and their physical interaction is required for normal SRY expression and Sertoli cell differentiation in normal testis determination (Tevosian *et al.* 2002, Bouma *et al.* 2007). Transgenic mouse fetuses homozygous for *Fog2*^{-/-} null mutation or a *Gata4*^{ki/ki} (a V217G amino acid substitution) mutation demonstrate that *Sry* expression level is significantly reduced in *Fog2*^{-/-} XY gonads and Sertoli cell differentiation/testicular tissue development is absent in *Fog2*^{-/-} XY and *Gata4*^{ki/ki} XY gonads (Tevosian *et al.* 2002, Manuylov *et al.* 2011). Recent study on the mutations in the *FOG2* gene in humans has revealed that mutant FOG2 proteins failed to directly interact with GATA4, and thus lead to anomalies of human sex determination and complete gonadal dysgenesis (Bashamboo *et al.* 2014). Three major Sertoli cell-specific genes critical for normal Sertoli cell differentiation (*Sox9*, *Amh*, and *Dhh*) and three genes encoding androgen biosynthetic enzymes (*P450scc*, *3βHsd*, and *P450c17*) during the steroidogenic program are absent in the *Fog2*^{-/-} XY and *Gata4*^{ki/ki} XY gonads, indicating that GATA4 and FOG2 physical interactions are

required for Sertoli cell development and Leydig cell maturation (Fig. 1C; Tevosian *et al.* 2002).

On the C57BL/6J (B6) genetic background, both gonadal phenotypes of mice lacking *Gadd45γ* and mitogen-activated protein kinase kinase kinase 4 (*Map3k4*) exhibit apparent XY gonadal sex reversal and identifiable ovaries and oviduct structures in XY gonads, which is caused by disruption of *Sry* expression in embryonic gonadal somatic cells during sex determination (Bogani *et al.* 2009, Warr *et al.* 2012). Gain- and loss-of-function experiments of *GADD45γ* and *MAP3K4* demonstrate *GADD45γ* binds to the N-terminus of *MAP3K4* and mediates the activation of *MAP3K4*, and then *MAP3K4* activates p38α and p38β through *MAP2K*, finally resulting in the direct or indirect regulation of *SRY* expression in XY supporting cells (Bogani *et al.* 2009, Warr *et al.* 2012). Consistent with *GADD45γ* functions through the p38 MAPK signaling pathway, reporter assays and ChIP experiments implicate that *GATA4* is involved in the MAPK signaling and the regulation of *Sry* expression (Gierl *et al.* 2012). *MAP3K4* and *GADD45γ* can phosphorylate and transactivate *GATA4*, and then phospho-*GATA4* recognizes and binds to the *Sry* promoter region to regulate the expression of *Sry* in gonadal somatic cells (Gierl *et al.* 2012). More recently, overexpression of a functional *Map3k4* bacterial artificial chromosome in *T*-associated sex reversal (*Tas*) mice can rescue the normal spatial and temporal expression of *Sry* in the developing gonads (Warr *et al.* 2014). This evidence suggests that a p38 *MAP3K4* signaling cascade, including *GADD45γ*-p38 *MAPK*-*GATA4*-*SRY*, is involved in the regulation of normal *Sry* expression during sex determination.

Microassay, immunohistochemical, and ChIP analyses of mice deficient in chromobox homolog2 (*Cbx2*; also known as *M33*) implicates that disruption of *Cbx2* dramatically decreased *Sry* gene expression and *Cbx2* may directly or indirectly regulate the expression of *Sry* through binding to the promoter of *Sf1* or other unknown mechanisms (Katoh-Fukui *et al.* 2012). In humans, the loss-of-function mutations of *CBX2* result in male-to-female sex reversal, suggesting that *CBX2* may function upstream of *SRY* to repress the ovarian differentiation (Biason-Laubier *et al.* 2009). The expression levels of several transcription factors involved in gonadal development, such as *Sox9*, *Lhx9*, *Sf1*, *Dax1*, *Dmrt1*, are decreased by approximately twofold or more in the *Cbx2*-KO mouse gonads (Katoh-Fukui *et al.* 2012).

The insulin family of growth factors, including *Ir*, *Igf1r*, and *Irr*, are required for normal *Sry* expression and early stage of testicular differentiation (Nef *et al.* 2003).

Combined ablation of the insulin receptor (*Ir*) and the IGF type 1 receptor (*Igf1r*) in the developing genital ridges in mice lead to reduced numbers of somatic precursor cells before sex determination, a delayed upregulation of *Sry* and other male-specific genes and the absolute absence of testicular differentiation in XY KO mice (Pitetti *et al.* 2013).

Target genes of SRY in the bipotential gonad

Sox9 *Sox9*, a member of the SOX transcription factor family, has long been thought to be the direct target gene of SRY during mammalian sex determination. *Sox9* is initially expressed in the lateral side of the bipotential genital ridge at E11.5 and upregulated in the Sertoli cell precursors in the XY male gonad immediately after the onset of *Sry* gene expression (Kent *et al.* 1996, Chaboissier *et al.* 2004). Unlike *Sry*, *Sox9* expression is maintained in the mouse testis during fetal and adult life, indicating that *Sox9* can sustain its expression once initiated.

Loss-of-function mutations in human SOX9 cause autosomal male-to-female sex reversal and a skeletal malformation called campomelic dysplasia (CD), and these revealed that SOX9 is involved in bone formation and sex determination (Foster *et al.* 1994). Meanwhile, the duplication of SOX9 gene in an SRY-negative female-to-male sex reversal patients demonstrated that SOX9 plays a crucial role in male sex determination and differentiation (Huang *et al.* 1999). Ectopically expression of *Sox9* in mouse XX gonads is sufficient to induce testis development and suggest that SOX9 can substitute for SRY during the initiation of the male differentiation program (Vidal *et al.* 2001). Homozygous deletion of *Sox9* in developing mouse XY gonad leads to irregular sex cord formation, abnormal Sertoli cell differentiation, and reduced expression of male-specific makers *Amh* and *P450c* (Chaboissier *et al.* 2004, Barrionuevo *et al.* 2006).

In summary, there are at least three molecular genetic pathways to ensure the maintenance of *Sox9* and accumulation of *Sox9*-positive Sertoli cell population: cell-autonomous *Sox9* autoregulation, FGF9-mediated signaling, and prostaglandin D₂ (PGD₂)-mediated signaling from Sertoli cell (Kashimada & Koopman 2010).

Mutation, co-transfection and sex reversal studies lead to the identification of a 1.4 kb testis-specific enhancer of *Sox9* core element (TESCO), which is located 11–13 kb upstream of the mouse *Sox9* transcription start site and is highly conserved in mammalian genomes. SRY can act synergistically with SF1 to regulate *Sox9* through binding to multiple elements within the *Sox9* gonad-specific

enhancer, and then SOX9 itself, and SF1 can cooperatively bind to the enhancer for its maintenance after SRY expression is ceased (Sekido & Lovell-Badge 2008).

There is a proposed model for the SRY/SF1-mediated SOX9 transcriptional regulation in the developing genital ridge (Sekido & Lovell-Badge 2008, Kashimada & Koopman 2010, Sekido 2010). Before E10.5, SF1 may, alone or cooperating with other partners, bind to three putative SF1 consensus-binding sites located within the evolutionarily conserved 1.4 kb TESCO and initiates a very low expression level of *Sox9* in the bipotential genital ridge of both sexes (Fig. 3A). At E11.0, SF1 recruits SRY through direct protein–protein physical interactions to the three putative SRY-binding sites, and SRY–SF1 complex significantly upregulates the *Sox9* expression in the male gonad (Fig. 3B). After E12.5, the expression level of *Sox9* has accumulated and reached a critical threshold, *Sox9* can recognize and occupy the SRY-binding sites thus synergistically interacting with the CTD of SF1 to promote the maintenance of *Sox9* by autoregulation after *Sry* expression is ceased (Fig. 3C). Meanwhile, other independent positive feedback loops also begins to work at this time point.

Previous studies show that loss of fibroblast growth factor 9 (*Fgf9*) results in male-to-female sex reversal and the nuclear localization of FGFR2 coincides with the initiation of *Sry* expression and overlaps with *Sox9* expression during the early differentiation of Sertoli cells (Schmahl *et al.* 2004). Gain- and loss-of-function experiments suggest that *Fgf9* functions in a feed-forward loop to upregulate *Sox9* expression and expand Sertoli cell precursors. *Fgf9* is essential for the maintenance of the expression of *Sox9*, and SOX9 is required for the upregulation of *Fgf9* in XY gonads (Kim *et al.* 2006).

In vivo experiments such as electrophoretic mobility shift assay (EMSA) and ChIP demonstrate that SOX9 recognizes and binds to the paired SOX-binding sites within the *Pgd2* promoter and then regulates the expression of *Pgd2* to ensure the sufficient Sertoli cells differentiation during normal sex development (Wilhelm *et al.* 2007a,b). *L-Pgds*-knockout XY mouse gonads show a significantly reduced expression level of *Sox9*, abnormal nuclear translocation of SOX9, and a delay in testicular organogenesis (Moniot *et al.* 2009). Autocrine or paracrine PGD₂ signaling, independent of FGF9-mediated signaling, can act as an amplification system of *Sox9* expression, promoting the nuclear localization of SOX9 via its cAMP-PKA phosphorylation and initiates subsequent Sertoli cell differentiation (Malki *et al.* 2005, Moniot *et al.* 2009).

Figure 3

Three emerging genetic pathways involved in the initial expression and maintenance of *Sox9* in Sertoli cells. (A) Before E10.5, SF1 binds to three putative SF1 binding sites located within the 1.4 kb TESCO and initiates a very low level expression of *Sox9* in the bipotential gonad. (B) At E11.0, SF1 recruits SRY through direct physical interactions with the three putative SRY-binding sites, and SRY-SF1 complex significantly activate the *Sox9* expression in the male gonad. (C) After E12.5, the expression level of *Sox9* reaches a critical threshold, *Sox9* can occupy the SRY-binding site, thus synergistically interacts with SF1 to promote the maintenance of *Sox9* by

autoregulation after SRY expression has been ceased. At the same time, other independent positive feedback loops, including FGF9-mediated signaling and PGD₂-mediated signaling, also begin to function. To date, at least three genetic pathways modulate the initial expression and maintenance of *Sox9* in Sertoli cell population: cell-autonomous *Sox9* autoregulation, FGF9-mediated signaling, and PGD₂-mediated signaling from Sertoli cells. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0018>.

Conserved genes in the mammalian ovary-determining pathway

Wingless-related MMTV integration site family, member 4

Wingless-related MMTV integration site family, member 4 (*Wnt4*) is initially expressed along the length of the mesonephros in the mesenchyme at E9.5–10.5, maintains its expression in the female gonad, is downregulated in the male gonad at E11.5, and is finally restricted to the mesenchymal cells surrounding the Müllerian duct epithelium in the bipotential genital ridge at E12.5 (Binnerts *et al.* 2007). Sex reversal phenotypes in the female reproductive system of the *Wnt4*-mutant mouse implies that *Wnt4* is essential in the formation of Müllerian duct, inhibition of Leydig cell precursor differentiation, differentiation of the interstitial cell lineage, and development of normal oocyte (Vainio *et al.* 1999).

Examination of XX gonads from *Wnt4*^{-/-} and *Fst*^{-/-} mutant mice revealed that *Wnt4* acts upstream of Follistatin (*Fst*) to regulate the formation of the coelomic

vessel and to maintain germ cell survival during ovary development in the female reproductive system (Yao *et al.* 2004). Loss of function of *Wnt4* leads to the transiently upregulation of both *Sox9* and *Fgf9* in the absence of *Sry*, indicating a genetic antagonism specifically between *Fgf9* and *Wnt4*, *Fgf9* functions to downregulate *Wnt4* in WT XY gonad. *Wnt4* can also downregulate *Fgf9* in normal XX gonad (Kim *et al.* 2006).

Overexpression of *Wnt4* in mice represses the SF1 and β -catenin synergism through disrupting the accumulation of β -catenin at SF1-responsive elements within multiple steroidogenic promoters, and *in vivo* studies show that the WNT signaling pathway antagonizes testosterone synthesis in adrenocortical and Leydig cell lines and blocks male development in embryonic ovaries (Jordan *et al.* 2003).

Forkhead box L2

Loss-of-function mutations in the *Foxl2* gene in human female patients were identified, which led to autosomal

dominant disorder blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) and premature ovarian failure in adult ovaries (Uhlenhaut & Treier 2006). Female *Foxl2*^{-/-} mice display premature ablation of the primordial follicle pool, inhibition of ovarian granulosa cell differentiation, and a complete absence of secondary follicles (Ottolenghi *et al.* 2007). Moreover, male-specific genes in male sex determination, including *SOX9*, *FGF9*, *FGFR2*, *SF1*, and *GATA4*, are ectopically activated in *Foxl2*^{-/-} ovaries, indicating that the forkhead transcription regulator *FOXL2* functions as a conserved repressor of the genetic cascade for male sex determination in mammals (Ottolenghi *et al.* 2007). Furthermore, cell-autonomous transdifferentiation of granulosa cells into Sertoli-like cells in the absence of oocyte function and reprogramming of theca cells into Leydig-like cells in XX *Foxl2*^{-/-} conditional mutant mice strongly suggest that *FOXL2* inhibits the transdifferentiation of an adult ovary to a testis (Uhlenhaut *et al.* 2009).

R-spondin 1

The disruption of *RSPO1* in four 46,XX testicular DSD patients leads to the identification of *RSPO1* as an ovary-determining gene during the early stage of gonad development (Parma *et al.* 2006). In human, *RSPO1* robustly expresses in the developing ovary from 6 to 9 weeks post conception, and homozygous mutations in *RSPO1* result in a recessive syndrome identified by complete female-to-male sex reversal (Parma *et al.* 2006, Tomaselli *et al.* 2011). Meanwhile, *Rspo1*-null (*Rspo1*^{-/-}) XX mice exhibit internal pseudohermaphroditism in the genital ridge, reduced numbers of follicles and oocytes depletion, and disrupted regression of the Wolffian duct in the female ovaries (Tomizuka *et al.* 2008). In XX *Rspo1*-deficient gonads, the germ cell proliferation decreases and XX fetal germ cell meiosis is largely disrupted (Chassot *et al.* 2011).

Dax1

Duplications of *DAX1* on chromosome Xp21 in XY individuals caused dosage-sensitive sex reversal (DSS) syndrome in humans, and transgenic XY mice carrying extra copies of *Dax1* gene result in downregulation of *Sry* and a delay in testis development (Swain *et al.* 1998). In the *Dax1*^{-/-} deficient XY mice, Sertoli cells and fetal Leydig cells are identifiable and appear to function normally, but are disorganized in the *Dax1*-deficient mice testis. Meanwhile, mutations of the *Dax1* gene are associated with abnormal peritubular myoid cell differentiation and primary testicular dysgenesis (Meeks *et al.* 2003).

Synergisms between female sex-determining genes in female sex development

Rspo1 is a candidate female-determining gene, and *Rspo1* regulates canonical WNT signaling pathways through mediating DKK1–Kremen1 interaction on the cell surface with increased expression levels of LRP6 (Binnerts *et al.* 2007). Current studies on *Rspo1*-deficient mice, in conjunction with the findings on *Wnt4*-null mice, show there is a remarkable resemblance between phenotypic features of *Rspo1*^{-/-} and *Wnt4*^{-/-} XX mice, indicating *Rspo1* is responsible for *Wnt4* expression in XX gonads and both of them participate in the suppression of the male sex determination pathway and maintain female germ cell survival (Chassot *et al.* 2008, Tomizuka *et al.* 2008).

Fst is an extracellular glycoprotein essential for female sex determination and early ovarian development. The female-specific gene *Fst* acts as a downstream target of WNT4 signaling pathway and involves the inhibition of testis-specific coelomic blood vessel (Yao *et al.* 2004). Recent observations in *Foxl2*-null mice have confirmed that *FOXL2* and *BMP2* cooperatively stimulate *Fst* expression in the developing ovary, and shed new light on precise mechanisms regulating *Fst* expression during ovarian development. *Wnt4* is a crucial regulator of initial expression of *Fst* in stroma differentiation and germ cell survival; *FOXL2* and *BMP2* are required to maintain *Fst* expression at the time of follicle formation (Kashimada *et al.* 2011a,b). *Wnt4* and *Foxl2* are independently expressed in ovarian somatic cells and the combined ablation of *Wnt4* and *Foxl2* leads to complete XX sex reversal (Ottolenghi *et al.* 2007). Taken together, there is a synergistic interaction and complementary association between *Wnt4* and *Foxl2* in the regulation of *Fst* expression in a time-dependent manner.

Detail phenotypic comparisons of *Foxl2*^{-/-} and *Rspo1*^{-/-} XX embryonic gonad suggest that *Rspo1* expression is independent of *Foxl2* expression and *Foxl2* expression is partially related to the *Rspo1*/β-catenin signaling pathway. Furthermore, XX *Foxl2*^{-/-} *Rspo1*^{-/-} double-mutant gonads are more masculinized when compared with XX *Rspo1*^{-/-} gonads, indicating that *Rspo1* and *Foxl2* function synergistically to ensure normal ovarian differentiation (Auguste *et al.* 2011).

Taken together, *Wnt4*, *Rspo1*, and *Foxl2* are crucial female sex-determining genes during the female sex development, and they have common roles in repressing testis differentiation (Table 1). The female sex determination pathways may involve two independent molecular pathways, the *RSPO1*-WNT4/β-catenin pathway and the

FOXL2 pathway, which regulate ovarian differentiation in the early steps of female sex development. *Rspo1* functions as the activator of the canonical WNT4/ β -catenin pathway to promote the female ovarian development and fetal germ cells meiosis.

Intercellular signaling mechanisms

Anti-Müllerian hormone

AMH, also known as Müllerian inhibiting substance, is a member of the TGF- β superfamily growth factors. During early steps of male sex determination, AMH is secreted in Sertoli cells and causes a gradient of cranial to caudal degeneration of the Müllerian duct, which will develop as the internal genitalia in female animals.

The *Amh* gene expression already begins around E11.5 in mouse gonads, coinciding with overexpression of *Sox9* in Sertoli cells, and there is a narrow time window between E13.5 and 14.5 for AMH functions on Müllerian duct regression in normal XY male gonads (Münsterberg & Lovell-Badge 1991, Gao *et al.* 2006).

Amh gene is an established target gene of SOX9 during embryonic gonad development. SOX9 can recognize the canonical SOX-binding site within the 180 bp proximal promoter region of *Amh* gene, and SF1 reinforces this regulation through a direct protein–protein interaction with SOX9 (Barbara *et al.* 1998). *In vitro* assays also established that SOX8 and SF1 can cooperatively interact with each other via the HMG domain and directly bind to the SOX-binding site in the *Amh* promoter to enhance *Amh* expression in a lower efficiency compared with SOX9 (Schepers *et al.* 2003). Consistent with those *in vitro* studies, combined ablation of *Sox9* and *Sox8* in XY mouse testis leads to dramatically reduced expression levels of the *Amh* gene compared with single-mutant testes, indicating that *Sox9* and *Sox8* function redundantly for the initiation and maintenance of high expression level of *Amh* in embryonic and adult Sertoli cells (Barriónuevo *et al.* 2009).

Gata4 and *Amh* are coexpressed in Sertoli cells and the binding and transactivation activity of *Gata4* on *Amh*-180 bp promoter region reveal that GATA4 is a potential regulator of *Amh* gene in Sertoli cells (Viger *et al.* 1998, Tremblay *et al.* 2001). In another complementary mechanism, GATA4 can functionally interact with SF1 through protein–protein interaction when SF1 binds to the *Amh* promoter (Tremblay *et al.* 2001). A recent study has reported that a heterozygous missense mutation (p.Glycine 211→Arginine) in the N-terminal zinc finger

region of GATA4 associated with a family case of 46,XY DSD results in the disruption of synergistic activation of the *Amh* promoter by GATA4 and SF1 (Lourenço *et al.* 2011). GATA4 (p.G211Arg) can physically interact with SF1 but not synergize with SF1 to stimulate the expression of *Amh* gene. This suggests that the DNA-binding activity of GATA4 is indispensable for its function in the AMH signaling cascade (Lourenço *et al.* 2011). Female-specific gene *Dax1* represses the transcription synergism between GATA4 and SF1 during the activation of *Amh* gene expression in Sertoli cells through negatively interacting with SF1, leading to a SF1-dependent transcriptional inhibition (Tremblay & Viger 2001).

Prostaglandin D synthase/PGD₂ signaling

Prostaglandin D synthase (*Ptgds*) initially appears in both Sertoli cell lineages and prospermatogonia between E11.5 and E12.5, and is involved in the final step of biochemical synthesis of PGD₂ (Adams & McLaren 2002). PGD₂ acts as an autocrine factor to stimulate the cAMP-dependent protein kinase A (PKA), inducing phosphorylation of SOX9 S61 and S181 PKA sites and facilitating the nuclear translocation of SOX9 (Malki *et al.* 2005). Detailed gene expression patterns analyses revealed that Lipocalin-type *Ptgds* (*L-Ptgds*) is male-specific and initially expressed in the Sertoli cell lineage at E11.5, around the time point of sex determination (Malki *et al.* 2005). The ablation of *L-Ptgds* expression in the XY gonads leads to a delay in the nuclear import of SOX9 and significantly reduced the *Sox9* and *Amh* expression levels. Meanwhile, recent genetic studies have indicated that SOX9 can bind to the conserved SOX-binding site within the *L-Ptgds* gene promoter region and control the onset expression of *L-Ptgds* (Wilhelm *et al.* 2007a,b). Moreover, conditional deletion of *Sox9* in Sertoli cells also dramatically down-regulates the *L-Ptgds* in the mutant XY gonads, indicating that maintenance of *L-Ptgds* is also dependent on SOX9 (Moniot *et al.* 2009). Taken together, PTGDS/PGD₂ signaling pathway forms a positive regulatory feedback loop with the *Sox9* gene, whereby PGD₂ can reinforce the expression of *Sox9* during the later stage of sex determination and SOX9 can initiate and maintain the *L-Ptgds* gene expression.

Vanin-1

Vanin-1 encodes a glycosylphosphatidylinositol-linked membrane-anchored pantetheinase, which is expressed in a male-specific manner in the Sertoli cells and Leydig

cells concordant with *Sf1* and *Sox9* (Bowles *et al.* 2000). *In vitro* reporter assays suggest that SF1 and SOX9 can bind to the *Vanin-1* proximal promoter and directly co-activate the mouse *Vanin-1* expression (Wilson *et al.* 2005).

The delicate balance between testis- and ovary-determining pathways

Reciprocal regulation of *Dax1* and *Sf1* in the modulation of expression of *Sry*, *Sox9*, and *Amh* genes

The spatial and temporal expression patterns of *Dax1* and *Sf1* coincide exactly in the XY bipotential genital ridge during the critical time of testis cord formation, and combined loss of function of both *Dax1* and *Sf1* in XY mouse gonads indicate DAX1 and SF1 function cooperatively to induce *Amh* expression, early Sertoli cell differentiation, and Leydig cell development (Park *et al.* 2005; Fig. 4A).

Genome scanning has identified a highly conserved SF1 consensus-binding site 4 kb upstream of *Dax1* and three proximal SF1 consensus-binding sites located close to the start site of transcription. *In vivo* studies of the regulation of *Dax1* implicates that SF1 is required for initial expression of *Dax1* during early gonad formation, but not for the maintenance of the expression at later stages of testis differentiation (Gummow *et al.* 2006).

In humans, XY individuals carrying duplications of *DAX1* on chromosome Xp21 lead to DSS syndrome and weak expression of *SRY* (Swain *et al.* 1998). Overexpression of *Dax1* in the XY gonads of *Dax1* transgenic mice also reduced the expression level of *Sox9* and the activity of the TES in XY gonads, and DAX1 repressed the cooperative activation of the TES element by *SRY* and SF1 in a dose-dependent manner (Ludbrook *et al.* 2012).

Wt1 and *Sf1* were co-expressed in embryonic Sertoli cells and WT1 (–KTS) physically associates with SF1 to modulate the expression of *Amh* during mammalian sexual differentiation. DAX1 antagonizes synergy between SF1 and WT1 through a direct interaction with SF1 (Fig. 4B; Nachtigal *et al.* 1998).

Balanced antagonistic signals between FGF9 and WNT4 in the bipotential gonad

Recent genetic studies have reveal that the gonadal differentiation is regulated by mutually antagonistic signals between the FGF9/SOX9 and WNT4 signaling pathways (Fig. 4A; Kim *et al.* 2006). The onset of *Sry* expression leads to an upregulation of *Sox9* and an

initiation of the male sex determination pathway, which in turn reinforces the expression of *Fgf9* and increased PGD₂ synthesis. The proteins FGF9 and PGD₂ can interact with *Sox9* and form two independent feed-forward loops to maintain the expression of *Sox9* and finally direct the differentiation of somatic cell lineages into Sertoli cells.

In the cultured XX gonads, exogenous *Fgf9* results in the downregulation of *Wnt4* (Kim *et al.* 2006), and the deletion of either *Fgf9* or its receptor *Fgfr2* in XY gonads lead to completely male-to-female sex reversal and significantly elevated expression level of *Wnt4*. Meanwhile, in the absence of both *Wnt4* and *Fgf9/Fgfr2* in XY-mutant mice, the deletion of *Wnt4* can rescue both the XY *Fgf9*^{−/−} and *Fgfr2*^{−/−} male-to-female sex reversal phenotypes, indicating that FGF9 signaling is required for the inhibition of the ovary-promoting gene *Wnt4* and subsequent female ovary differentiation (Jameson *et al.* 2012).

Upregulation of *Sox9* and *Fgf9* and the absence of Müllerian duct formation were observed in XX *Wnt4*^{−/−} gonads (Bernard & Harley 2007). The deletion of *Fgf9* cannot rescue the XX female-to-male phenotypes in the XX *Wnt4*^{−/−} gonads, indicating that there is an asymmetric relationship between *Fgf9* and *Wnt4* in the sex determination molecular cascade (Jameson *et al.* 2012).

The lifelong opposing interactions between *Sox9* and *Foxl2*

A comprehensive study of *Sox9*- and *Sox8*-mutant testes indicates that ovarian marker *Foxl2* begins to be expressed in Sertoli cells shortly after the combined deletion of *Sox9* and *Sox8* and the increasing number of *Foxl2*-positive cells in XY gonads during the later developmental stage of testis formation, suggesting a progressive transdifferentiation of Sertoli cells into female-specific cells (Georg *et al.* 2012).

Conditional deletion of *Foxl2* during folliculogenesis in mature female mice causes ectopic expression of *Sox9* in follicles and postnatal female-to-male sex reversal in the XX gonad, together with the cell-autonomous and oocyte-independent reprogramming of granulosa cells into Sertoli-like cells (Uhlenhaut *et al.* 2009). *In vitro* studies also show that *Foxl2* can synergize with estrogen receptor 1 (ESR1) to negatively modulate the expression of *Sox9* in postnatal ovaries through direct physically interaction with the TESCO element of *Sox9* (Uhlenhaut *et al.* 2009). *Foxl2* also specifically interacts with SOX9/SF1 and negatively modulates the transactivation of *Cyp17* and *Cyp26b1*, which function in the balance of pro-androgen and pro-estrogen (Park *et al.* 2010, Kashimada *et al.* 2011a,b).

Figure 4

Delicate balance between male and female sex-determining pathways. (A) In the mammalian bipotential gonad, the fate of the genital ridge to development into a testis or an ovary is balanced by the opposing male and female sex-determining genes. i) FGF9 signaling inhibits the ovary-promoting gene *Wnt4* and subsequent female ovary differentiation. *Wnt4* and downstream target β -catenin in turn repress the *Fgf9/Sox9* regulatory loop. ii) *Foxl2* specifically interacts with *Sox9/Sf1* and negatively modulate the transactivation of *Cyp17* and *Cyp26b1*, which are required for steroidogenesis in Sertoli cells. *Cyp26b1* also inhibits the local distribution of retinoic acid in the Sertoli cells and Leydig cells to control the germ cell fate (Kashimada *et al.* 2011a,b). *Foxl2* and *Esr1* can also cooperatively downregulate the expression of *Sox9*. *Foxl2* directly regulates the expression of aromatase during the ovarian development (Garcia-Ortiz

et al. 2009). iii) In the female sex determination pathway, *Rspo1* activates *Wnt4* expression in somatic cells and regulates the canonical β -catenin signaling pathway. (B) Reciprocal regulation of *Dax1* and *Sf1* in regulation of *Sry*, *Sox9*, and *Amh* expression. In the female development pathway, amounting numbers of DAX1 inhibit GATA4/SF1-, SRY/SF1-, and SF1/SOX9-mediated activation of *Tesco*, providing a mechanism for the SOX9-DAX1 antagonism. (C) Proposed conceptual model for the regulation of postnatal sex development by *DMRT1* in mammals. *DMRT1* activates a handful of male-specific genes, including *SOX9*, *SOX8*, and *PGD2*, and significantly reduces the expression of female-promoting genes, including Forkhead box L2 (*FOXL2*), *ESR1*, *WNT4*, and R-spondin 1 (*Rspo1*). Meanwhile, *FOXL2* and *DMRT1* exhibit opposing functions on the regulation of sex development. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0018>.

Cyp26b1, a retinoic acid-degrading enzyme, also regulates the local distribution of retinoic acid to control germ cell meiosis (Kashimada *et al.* 2011a,b).

Reciprocal antagonism between *Sox9* and *Foxl2* is an active permanent process throughout the whole life, and *Sox9* and *Foxl2* repress each other's functions in order to maintain the male or female fate of supporting cell lineages during later stages of sex development (Fig. 4A; Uhlenhaut *et al.* 2009, Veitia 2010).

The critical crosstalk between the Rspo1/ β -catenin signaling pathway and the SOX9 network

Targeted deletion of *Rspo1* in XX mice reveal that *Rspo1* is required for *Wnt4* expression in somatic cells and functions as a crucial activator of the canonical β -catenin signaling pathway in female sex determination (Chassot *et al.* 2008). The activation of β -catenin signaling pathway abolishes the establishment of *Sox9* in supporting cell

precursors (Capel 2006). The ablation of *Rspo1* leads to upregulation of *Pgd2*, *Sox9*, and *Amh* expression in the absence of *Sry*, which can form a positive feedback loop to reinforce each other's expression (Chassot *et al.* 2008; Fig. 4A).

Antagonisms between *Dmrt1* and ovary-specific genes in adult gonads

Recent genetic studies of *Dmrt1* null-mutant XY adult mice have revealed that loss of the *Dmrt1* in mouse Sertoli cells causes dramatically decreased expression of several male-specific genes, including *Sox9*, *Sox8*, and *Ptgds*, and significantly robust expression of female-promoting genes, including *Foxl2*, *Esr1/2*, *Wnt4*, and *Rspo1* (Fig. 4C; Matson *et al.* 2012). Loss-of-function of *Dmrt1* that leads to postnatal Sertoli cells transdifferentiated into granulosa cells indicate that *Dmrt1* play a pivotal role in the maintenance of mammalian testis determination long after the sex determination stage in male and female (Matson *et al.* 2012). Conditional deletion of *Foxl2* in adult ovarian follicles in mice leads to significant upregulation of *Dmrt1* suggesting that *Foxl2* may directly repress *Dmrt1* transcription during folliculogenesis in sexually mature female mice, and *Foxl2* and *Dmrt1* exhibit opposing functions in the regulation of sex development (Uhlenhaut *et al.* 2009).

Concluding remarks and future perspectives

Recent genetic studies of the cellular, genetic, and molecular aspects of mammalian sex determination have shed a new global insight into the gene network and molecular pathway of sex development. In mammals, sex determination is the developmental decision that directs the bipotential genital ridge to differentiate into either a testis or an ovary. During male sex determination, SRY is the master regulator of male sex-determining cascade, and its crucial downstream target gene *Sox9* is indispensable for the differentiation of the Sertoli cell lineage and male-specific sex determination molecular cascade. However, in mammals, the female sex determination pathway has long been considered as the passive 'default' pathway of gonadal development in the absence of SRY. Recent studies have challenged the female default pathway by the presence of *Rspo1*, *Wnt4*, *Foxl2*, and *Dax1* as female-specific sex-determining genes during ovarian development. Several lines of evidence suggest the prevailing view that female fate is the 'default' state that is imperfect, and the female sex determination

pathway also requires an active involvement of female sex-determining genes.

Accumulating evidence suggests that the genetic network underlying sex determination and sex differentiation is far more complex and dynamic than portrayed so far. Male-specific and female-specific sex-determining genes antagonize each other's function to establish and maintain the different supporting cell lineage fates during sex determination and throughout adulthood.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Author contribution statement

Z-Y S and W-X Y conceived of and authored the manuscript.

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