Molecular mechanisms involved in mammalian primary sex determination

Zhen-Yu She and Wan-Xi Yang
The Sperm Laboratory, College of Life Sciences, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou 310058, China

Correspondence should be addressed to W-X Yang
Email wxyang@spermlab.org

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

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 lead 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 (NR0B1), 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

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<td>Emx2</td>
<td>Transcription factor</td>
<td>Aberrant tight junction assembly, failure in genital ridge formation (LOF)</td>
<td>Kusaka et al. (2010)</td>
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<td>Gata4</td>
<td>Transcription factor</td>
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<td>Nuclear receptor</td>
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<td>Park et al. (2005) and Lin et al. (2007)</td>
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<td><strong>Genes involved in the regulation of SRY expression during primary sex determination</strong></td>
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<td>Gata4/Fog2</td>
<td>Transcription/cofactor</td>
<td>Apparent XY gonadal sex reversal (LOF)</td>
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<td>Gadd45γ</td>
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<td>Kinase</td>
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<td>Bogni et al. (2009)</td>
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<td>Cbx2</td>
<td>Transcription factor</td>
<td>–</td>
<td>Blason-Lauber et al. (2009) and Katoh-Fukui et al. (2012)</td>
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<td>Ir, Irr, Igf1r</td>
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<td>Nef et al. (2003)</td>
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<td>SRY</td>
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<td>Turner syndrome, Klinefelter syndrome, XY sex reversal (LOF)</td>
<td>Ford et al. (1959), Jacobs &amp; Strong (1959), Koopman et al. (1989) and Gubbay et al. (1990)</td>
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<td>Transcription factor</td>
<td>Campomelic dysplasia XY sex reversal (LOF)</td>
<td>Foster et al. (1994), Huang et al. (1999), Vidal et al. (2001), Chaboissier et al. (2004) and Barriouveau et al. (2006)</td>
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<td>Amh</td>
<td>Hormone</td>
<td>XY sex reversal (LOF)</td>
<td>Kim et al. (2006) and Jameson et al. (2012)</td>
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<td>Growth factor</td>
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<td>Matson et al. (2012)</td>
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<td>Dmrt1</td>
<td>Transcription factor</td>
<td>XY gonadal dysgenesis, XY sex reversal (LOF)</td>
<td>Postnatal feminization in XY mice, defective seminiferous tubule (LOF)</td>
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References:
- Kusaka et al. (2010)
- Lourenc¸o et al. (2011)
- Manuylov et al. (2011)
- Hu et al. (2013)
- Kreidberg et al. (1993)
- Hammes et al. (2001)
- Gao et al. (2006)
- Birk et al. (2000)
- Park et al. (2005)
- Lin et al. (2007)
- Tevosian et al. (2002)
- Gierl et al. (2012)
- Warr et al. (2012)
- Bogni et al. (2009)
- Blason-Lauber et al. (2009)
- Katoh-Fukui et al. (2012)
- Nef et al. (2003)
- Ford et al. (1959)
- Jacobs & Strong (1959)
- Koopman et al. (1989)
- Gubbay et al. (1990)
- Foster et al. (1994)
- Huang et al. (1999)
- Vidal et al. (2001)
- Chaboissier et al. (2004)
- Barriouveau et al. (2006)
- Kim et al. (2006)
- Jameson et al. (2012)
- Matson et al. (2012)
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 homebox 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein function</th>
<th>Human syndrome</th>
<th>Mouse models</th>
<th>Gain- and loss-of-function phenotypes</th>
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<td>Wnt4</td>
<td>Transcription factor</td>
<td>Ambiguous genitalia (GOF)</td>
<td>Jordan et al. (2003) and Yao et al. (2004)</td>
<td>Failure in the formation of coelomic vessel and germ cell, male XX sex reversal (LOF)</td>
</tr>
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<td>β-catenin</td>
<td>Growth factor</td>
<td>Palmoplantar hyperkeratosis, skin carcinoma, complete XX sex reversal (LOF)</td>
<td>Chassot et al. (2008) and Parma et al. (2006)</td>
<td>Premature ovarian failure, addition of the primordial follicle (GOF), XX sex reversal (LOF)</td>
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<td>Rspo1</td>
<td>Transcription factor</td>
<td>XX sex reversal (LOF)</td>
<td>Swain et al. (1998) and Meeks et al. (2003)</td>
<td>XX sex reversal (LOF)</td>
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<td>FoxL2</td>
<td>Nuclear receptor</td>
<td>XX sex reversal (LOF)</td>
<td>Chassot et al. (2008)</td>
<td>XX sex reversal (LOF)</td>
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Table 1 Continued
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 aristless-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 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).

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 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, 3\(^\alpha\)HSD, 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.
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 for robust expression of Dmrt1. 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 (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

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**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, and 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 (Impβ) 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 GADD45γ-p38 MAPK-GATA4-SRY, is involved in the regulation of normal SRY expression during sex determination. The insulin receptor tyrosine kinase family, comprising Ir, Irg, Irgf1r, 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 & Love-ll-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 Gata4kiekie (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 Gata4kiekie 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 Gata4kiekie 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 Gadd45α 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 GADD45α and MAP3K4 demonstrate GADD45α 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 GADD45α 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 GADD45α 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 GADD45α-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-Lauber 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 Iri, Igf1r, and Irs, 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. 2004).

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). Ectopic 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 gonads leads to irregular sex cord formation, abnormal Sertoli cell differentiation, and reduced expression of male-specific makers Amh and P450sc (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, FGFR9-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 of SRY/SF1 and then SF1 recruits SRY to initiate Sox9 expression and overlaps with Sox9 expression during the early differentiation of Sertoli cells (Wilhelm et al. 2007a,b). L-Pgd2-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).
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<sup>K<sub>K</sub></sup>/K and Fst<sup>K<sub>K</sub></sup>/K 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 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, Fgf2, 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 RSP01 in four 46,XX testicular DSD patients leads to the identification of RSP01 as an ovary-determining gene during the early stage of gonad development (Parma et al. 2006). In human, RSP01 robustly expresses in the developing ovary from 6 to 9 weeks post conception, and homozygous mutations in RSP01 result in a recessive syndrome identified by complete female-to-male sex reversal (Parma et al. 2006, Tomaselli et al. 2011). Meanwhile, Rs0p1-null (Rs0p1−/−) 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 Rs0p1-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 appeal 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 RSP01-WNT4/β-catenin pathway and the
FOXL2 pathway, which regulate ovarian differentiation in the early steps of female sex development. 

**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 *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 (Barrionuevo 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→Argnine) 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 panetheinase, 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 SFI 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 SFI 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 SFI 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 SFI consensus-binding site 4 kb upstream of Dax1 and three proximal SFI consensus-binding sites located close to the start site of transcription. In vivo studies of the regulation of Dax1 implicates that SFI 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 (Gumimmow et al. 2006).

In humans, XY individuals carrying duplications of DXAX1 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 SFI in a dose-dependent manner (Ludbrook et al. 2012).

Wt1 and SFI were co-expressed in embryonic Sertoli cells and WT1 (−KTS) physically associates with SFI to modulate the expression of Amh during mammalian sexual differentiation. DAX1 antagonizes synergy between SFI and WT1 through direct physical interaction with SFI (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 granulose 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-oestrogen (Park et al. 2010, Kashimada et al. 2011a,b).
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).

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.

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 lineages during later stages of sex development.
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, Est1/2, Wnt4, and Rsps1 (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 Rsps1, 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

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