The Genetics of Disorders of Sex Development in Humans

Thomas Ohnesorg^a Eric Vilain^b Andrew H. Sinclair^a

^aMurdoch Children's Research Institute and Department of Paediatrics, The University of Melbourne, Royal Children's Hospital, Melbourne, Vic., Australia; ^bDepartment of Medical Genetics, University of California Los Angeles, Los Angeles, Calif., USA

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Abstract

One of the defining events during human embryonic development with the most far-reaching effects for the individual is whether the embryo develops as male or female. The crucial step in this process is the differentiation of the bipotential embryonic gonads into either testes or ovaries. If the embryo inherits X and Y sex chromosomes, the Y-linked *SRY* (sex determining region in Y) gene initiates a network of genes that results in a functional testis and ultimately a male phenotype. By contrast, in an embryo with 2 X chromosomes, the undifferentiated gonad develops as an ovary resulting in a female phenotype. Perturbation of any of the genes in either the testicular or ovarian developmental pathway can result in individuals with disorders of sex development. In this review, we provide a summary of known components of testicular or ovarian pathways and their antagonistic actions and give a brief overview of new technologies currently used to identify the missing pieces of the sex development network.

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The development of the gonads differs from other organs as they have the potential to differentiate into 2 functionally distinct organs, testes or ovaries. Once functional gonads are formed, hormones released by either testes or ovaries lead to the development of sexual characteristics and eventually to a fertile male or female individual.

In humans and other mammals, sex is determined genetically at fertilization by the sex chromosome constitution. In most mammals (with the exception of monotremes and few species of mole voles and spiny rats), 2 X chromosomes result in ovary (female) development while the inheritance of X and Y chromosomes results in testis (male) development. Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical. DSDs range from mild forms of hypospadias to complete sex reversal. Hypospadias occurs with a high frequency of 1 in 250 live births while the incidence of gonadal anomalies is lower and estimated to be 1 in 4,500 live births [Baskin et al., 2001; Hughes et al., 2006].

Patients with DSD have been critical to the discovery of genes responsible for gonadal development. In 1990, the analysis of 46,XX testicular DSD (female-to-male sex reversal) patients with translocation of Y chromosome material to one of the X chromosomes led to the discovery of the sex determining region Y (*SRY*) gene, which causes

the undifferentiated embryonic gonad to develop as a testis [Sinclair et al., 1990]. In the absence of the Y chromosome (and absence of *SRY*), gonads develop as ovaries. Consequently, the crucial event in sex determination is whether or not a testis develops. It is thought that the role of *SRY* in this pathway is to initiate a hierarchy of genes eventually leading to testis development.

Since Jost's discovery in the 1940s that the removal of embryonic gonads of rabbits leads to a female phenotype (regardless of the chromosome constitution), the female pathway was long thought to be a passive or 'default' pathway. However, mounting evidence contradicts this hypothesis as it is becoming clearer that components of both male and female differentiation pathways act by suppressing components of the opposing pathway to ensure proper development and maintenance of the gonads (see below).

Since the discovery of *SRY* as the master switch in male development, several autosomal genes with important functions in either male or female development have been identified. However, there are still extensive gaps in our understanding of the complex genetic networks leading to either functional testes or ovaries. At present, the majority of DSD cases cannot be explained. This strongly suggests that important components of the sex development pathways are yet to be discovered. Recent technological advances like SNP arrays (to detect copy number variation in the genome) and massively parallel sequencing are likely to lead to new and important findings allowing us to better elucidate the mechanism of human sex determination and differentiation.

Genetic diagnoses for DSDs have become the first step which families can use to build a future and address their many anxieties. The advances in genomic technologies translate to clinical diagnoses and provide a much-needed management guide to patients, families and practitioners. In addition, progress in genomic analyses has allowed better-defined categorizations of patients with DSDs and therefore better, evidence-driven outcome studies. In this review, we will mostly focus on genes involved in the first steps of gonadal sex determination, namely the formation of the undifferentiated gonad and the development of the testis or the ovary.

Formation of the Undifferentiated Gonad

At early stages of mammalian embryonic development, the embryos are sexually undifferentiated. The undifferentiated gonads derive from the intermediate mesoderm and arise as paired thickenings of the coelomic epi-

thelium on the ventromedial surface of the mesonephros [Sato, 1985]. A distinct feature of the gonads is that, in contrast to other organs, they can develop into 2 functionally different structures, testes and ovaries.

Genes Required for Early Undifferentiated Gonad Formation

Steroidogenic factor 1 (*NR5A1/SF1*) is a member of the orphan nuclear receptor family and a key transcription factor involved in the regulation of many steroidogenic enzymes [Val et al., 2003]. In mice, *Sf1* is expressed from the earliest stages in the urogenital ridge. *Sf1* is downregulated during ovarian development but shows continued expression throughout testicular development [Luo et al., 1994]. In the testis, *Sf1* expression is restricted to Sertoli and Leydig cells. However, its expression in all primary steroidogenic tissues, including the developing hypothalamus and pituitary gonadotropes, suggests a role in additional levels of the reproductive axis. Interestingly, *Sf1* knockout mice lack both adrenal glands and gonads. These mice develop as phenotypic females but die soon after birth due to adrenal failure [Luo et al., 1994]. In humans, mutations of *SF1* are known to cause 46,XY DSD gonadal dysgenesis, premature ovarian insufficiency and adrenal failure [Achermann et al., 1999; Lourenço et al., 2009]. Taken together, this data indicates a role for *SF1* in the formation of the undifferentiated embryonic gonads as well as in subsequent gonadal sex determination.

The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger protein. It can act as a transcriptional repressor or activator depending on the cellular or chromosomal context [Hohenstein and Hastie, 2006]. It is also known to be associated with a pediatric kidney cancer. Denys-Drash syndrome, characterized by renal failure and genital abnormalities, including 46,XY gonadal dysgenesis, can be caused by heterozygous mutations in the zinc finger domain of *WT1* [Pelletier et al., 1991]. A mutation in a splice donor site in *WT1* leads to the loss of a specific isoform of *WT1*, causing Frasier syndrome and associated 46,XY DSD gonadal dysgenesis [Barboux et al., 1997]. In the mouse, *Wt1* is expressed in the undifferentiated gonads at the same time as *Sf1*. *Wt1* null mice fail to develop both kidneys and gonads [Kreidberg et al., 1993]. *WT1* acts together with *SF1* early in urogenital development to ensure proper formation of the undifferentiated gonad [Wilhelm and Englert, 2002]. Mutations in *WT1* leading to 46,XY DSD gonadal dysgenesis in both Denys-Drash and Frasier syndrome patients as well as in knockout

mouse models indicate that *WT1* plays also a role in gonadal sex (testis) determination.

Another gene important for early gonadal development is *CBX2* (chromobox homolog 2, also known as *M33* in the mouse), which shows similarity to the *Drosophila* polycomb group of genes. Mice carrying a disrupted *Cbx2* gene show defects in adrenal, splenic and gonadal development associated with varying degrees of XY sex reversal [Katoh-Fukui et al., 1998]. Further analysis of these mice revealed reduced expression levels of several testis genes, including *Sry* [Katoh-Fukui et al., 2012]. Interestingly, a recent report describes a *CBX2* loss-of-function mutation in a case of 46,XY DSD gonadal dysgenesis with a completely normal female phenotype [Bisson-Laubert et al., 2009].

SRY (sex determining region on the Y chromosome) is a single exon gene that encodes a 79-amino-acid motif, the HMG box, which is capable of sequence-specific binding and bending of DNA [Giese et al., 1992]. Only the HMG box of *SRY* is conserved between mammalian species, suggesting that the *SRY* protein acts as an architectural transcription factor influencing the expression of its target genes by inducing conformational changes in the surrounding chromatin. *SRY* was isolated using DNA from sex-reversed patients who either carried 2 X chromosomes but formed testes and were phenotypically male (46,XX testicular DSD) or had XY chromosomes and were phenotypically female (46,XY DSD gonadal dysgenesis). Subsequent analysis of these patients showed that 80% of the 46,XX testicular DSD males had some Y chromosome material (including *SRY*) translocated onto one of their X chromosomes, and about 20% of the 46,XY DSD gonadal dysgenesis females had a loss-of-function mutation in the *SRY* gene [Sinclair et al., 1990]. Taken together, these findings confirmed that *SRY* was required for testis formation and male gonadal sex determination. Final proof came from the observation that female mice transgenic for the *Sry* gene developed as males with sterile testes [Koopman et al., 1991]. This demonstrated that *SRY* is the only Y-linked gene necessary and sufficient to initiate testis development and thus represents the master testis-determining gene in almost all mammals.

In mice, *Sry* appears only as a brief burst of expression just prior to morphological differentiation of the embryonic testis [Hanley et al., 2000]. Interestingly in humans, *SRY* continues to be expressed in the adult testis [Clépet et al., 1993]. In the mouse embryo, it is expressed in pre-Sertoli cells, and its main function appears to be the initiation of Sertoli cell differentiation. Pre-Sertoli cells stimulate the formation of testicular cords and Leydig cell

Table 1. Genes involved in human sex determination

Gene	Loss of function	Gain of function
<i>CBX2</i>	46,XY DSD	
<i>WT1</i>	46,XY DSD, Denys-Drash and Frasier syndrome	
<i>SRY</i>	46,XY gonadal dysgenesis	46,XX testicular DSD
<i>SOX9</i>	46,XY gonadal dysgenesis, usually with campomelic dysplasia	46,XX testicular DSD
<i>NR5A1</i> (<i>SF1</i>)	46,XY gonadal dysgenesis, usually with adrenal failure	
<i>NR0B1</i> (<i>DAX1</i>)	hypogonadotropic hypogonadism and congenital adrenal hypoplasia	46,XY gonadal dysgenesis
<i>DMRT1</i>	46,XY gonadal dysgenesis	
<i>ATRX</i>	46,XY DSD	
<i>MAP3K1</i>	46,XY gonadal dysgenesis	
<i>GATA4</i>	46,XY DSD	
<i>FOG2</i>	46,XY DSD	
<i>DHH</i>	46,XY DSD	
<i>FGF9</i>		46,XX testicular DSD
<i>WNT4</i>	46,XX testicular DSD	46,XY gonadal dysgenesis
<i>RSPO1</i>	46,XX testicular DSD with hyperkeratosis	

formation in the surrounding interstitium. This suggests that *Sry* acts as a switch toward Sertoli cell fate and testis differentiation, but is, at least in mice, not required for the maintenance or function of the testis.

Several transcription factors have been shown to positively regulate *Sry* expression, including *Wt1*, *Sf1/Nr5a1* and *Gata4* (GATA binding protein 4) (see below) [Hammes et al., 2001; Tevosian et al., 2002; Wilhelm and Englert, 2002]. As well as transcription factors, epigenetic regulation appears to play an important role in *Sry* expression as highlighted by a recent publication. The authors of this study showed that knockout of the H3K9 demethylase gene *Kdm3a* prevents expression of *Sry* and leads to male-to-female sex reversal [Kuroki et al., 2013].

Testis Development

Several autosomal genes are known to play a role downstream of *SRY* in testis development. Copy number variation and mutations in these genes are associated with DSD (table 1). Unlike the male Y-specific *SRY* gene, these genes are present in both sexes but show differential sex-specific expression.

SOX9 (*SRY*-related HMG box 9) is a transcription factor essential for both gonadal and skeletal development. It belongs to the same family of developmental regulators as *SRY*, the *SOX* proteins. In contrast to *SRY*, *SOX9* pos-

sesses both a DNA-binding HMG box and a transactivation domain. *SOX9* is expressed in a variety of fetal tissues including brain, testis and chondrocytes [Wright et al., 1995]. Mutations in *SOX9* are associated with campomelic dysplasia, a rare congenital skeletal malformation syndrome, characterized by anomalies of the ribs and vertebral column, bowing of the long bones and short stature. About 75% of 46,XY individuals with campomelic dysplasia develop as phenotypic females or present with ambiguous genitalia. Loss-of-function mutations in one allele of *SOX9* have been identified in patients with campomelic dysplasia and 46,XY DSD [Foster et al., 1994; Wagner et al., 1994]. This indicates that haploinsufficiency of *SOX9* is responsible for both conditions. Duplication of the chromosome 17q region, including *SOX9*, can also result in 46,XX testicular DSD, probably due to an increase in *SOX9* expression levels [Huang et al., 1999]. Similarly, in mice, *Sox9* gain-of-function in XX transgenic mice leads to testis development, whereas loss-of-function results in complete XY sex reversal [Vidal et al., 2001; Barrionuevo et al., 2006]. This suggests that, like *SRY*, *SOX9* is both necessary and sufficient for male development.

In the mouse, *Sox9* is upregulated specifically in pre-Sertoli cells suggesting its role is to induce Sertoli cell differentiation [Sekido et al., 2004]. *Sox9* is expressed in the embryonic gonad just after the onset of *Sry* expression but, unlike *Sry*, is expressed throughout embryogenesis and adult life. It has long been assumed that *SOX9* is a direct target of *SRY*. However, as the regulatory region of *SOX9* spans over more than a megabase of genomic DNA and the proximal promoter region could not be shown to contain a functional *SRY* binding site [Kanai and Koopman, 1999; Gordon et al., 2009], the *SRY*-*SOX9* relationship remained speculative. In 2008, Sekido and Lovell-Badge [2008] described an enhancer approximately 13 kb upstream of *Sox9* (testis-specific enhancer of *Sox9* core, TESCO). In mice, *SRY* binds synergistically with SF1 to this enhancer and induces upregulation of *Sox9*. After *SRY* expression ceases, *SRY* is replaced by *SOX9* at this enhancer to maintain its own expression [Sekido and Lovell-Badge, 2008]. However, this interaction has not been confirmed in other species. Furthermore, it is unlikely that this enhancer is the only region responsible for *SOX9*-regulated gonad expression. Recent studies show that rearrangements of genomic regions far upstream of *SOX9* can lead to sex reversal in humans [Benko et al., 2011; Cox et al., 2011; Vetro et al., 2011]. Current evidence suggests that the main function of *SRY* is the initiation of *SOX9* upregulation. In contrast to *SRY*, *SOX9*

is present in all vertebrates examined so far. This suggests that the crucial event in male development is the upregulation and maintenance of *SOX9* expression levels in the testis. While *SRY* is responsible for the initiation, other factors are required for the maintenance of *SOX9* expression (see below).

In mice, *SRY* is only expressed for a very brief interval, so other genes must ensure maintenance of *Sox9* expression at levels high enough for testis development. One of these genes is *Fgf9*, a member of the fibroblast growth factor family. FGFs are involved in many developmental processes in the embryo including cell migration, survival, differentiation, and proliferation. *Fgf9* is expressed at low levels in both sexes but becomes upregulated in the developing testis shortly after the onset of *Sry*. *Fgf9* null mice were shown to display XY gonadal dysgenesis with impaired Sertoli cell development [Colvin et al., 2001]. *SOX9* expression is not maintained at levels high enough to ensure testis development in these mice, leading to the expression of ovarian genes and male-to-female sex reversal. Current evidence suggests that *SOX9* upregulates *Fgf9*, which in turn triggers a *SOX9*/FGF9 positive feedback loop to maintain high levels of *Sox9* expression in the developing testis. It was shown that, in the testis, FGF9 exerts its function through its receptor FGFR2 [Schmahl et al., 2004]. Unsurprisingly, *Fgfr2* null mice are phenotypically very similar to *Fgf9* null mice [Bagheri-Fam et al., 2008]. In humans, single nucleotide variations in *FGFR2* have been identified in several hypospadias patients [Beleza-Meireles et al., 2007]. Furthermore, 2 recent reports of CNVs, a duplication of *FGF9* in a 46,XX male [Chiang et al., 2013] and a deletion of *FGFR2* in a 46,XY female with ambiguous genitalia [Tannour-Louet et al., 2010], suggest an involvement of both genes in human gonadal development. However, further research is required to confirm their causal role in human DSDs.

Desert hedgehog (*DHH*) is a member of the hedgehog gene family that encodes signaling molecules involved in the regulation of morphogenesis. *DHH* is the only member of this family that has been shown to play a role in testis development and fertility. In humans, homozygous missense mutations in *DHH* cause 46,XY complete or partial gonadal dysgenesis with or without minifascicular polyneuropathy [Umehara et al., 2000; Canto et al., 2004]. Although *Dhh* knockout mice are sterile and show lower levels of testosterone due to impaired Leydig cell function, they do not display sex reversal [Bitgood et al., 1996]. In mice, *DHH* is therefore thought to induce proper Leydig cell differentiation in the developing testis.

The *Drosophila dsx* (doublesex) gene and the *Caenorhabditis elegans mab-3* (male abnormal) gene encode transcription factors involved in sexual differentiation. Both these transcription factors bind their target DNA via a so-called DM domain. A search for DM-related genes in humans revealed, amongst others, the *DMRT1* (DM-related transcription factor 1) gene [Raymond et al., 1998]. Interestingly, expression of *DMRT1* was shown to be restricted to testes in humans and mice. Monosomy of the chromosome 9p region, including *DMRT1*, is associated with 46,XY DSD [Raymond et al., 1999]. However, *Dmrt1* null mice do not show male-to-female sex reversal. *DMRT1*'s main role appears to be in postnatal Sertoli and germ cell maintenance [Raymond et al., 2000]. Interestingly, in the chicken, knockdown of *DMRT1* leads to feminization of genetically male gonads. This suggests that in birds, which lack *SRY*, *DMRT1* has evolved as the critical testis-determining gene. It also indicates that *DMRT1* plays an important role in several aspects of vertebrate testicular development [Smith et al., 2009]. A recent report highlights the important function of *DMRT1* in maintenance of functional testes in adult life (see below).

AMH (anti-Müllerian hormone) is a member of the transforming growth factor-beta family and one of the first hormones produced by the developing testis. It is synthesized in Sertoli cells and causes regression of the Müllerian ducts, which in females develop as oviducts, uterus and upper vagina [Cate et al., 1986]. AMH is therefore required for sex-specific differentiation of the reproductive tract; however, lack of AMH does not affect testicular differentiation itself. In mice, *SOX9* was shown to be essential for initiating *Amh* expression, while *SF1* directly interacts with both *SOX9* and *WT1* to significantly upregulate *Amh* transcript levels in male embryos [Arango et al., 1999].

NR0B1/DAX1 (nuclear receptor subfamily 0, group B, member 1/DSS-AHC critical region of the X chromosome, gene 1) encodes an unusual orphan nuclear receptor with a ligand-binding domain but lacking the characteristic nuclear receptor zinc finger DNA-binding domain. Duplications of *DAX1* have been shown to cause 46,XY DSD [Baumstark et al., 1996; Sanlaville et al., 2004]. While loss-of-function mutations or deletions of the *DAX1* gene result in patients with adrenal hypoplasia congenita and hypogonadotropic hypogonadism, no effect on testicular development was observed [Muscatelli et al., 1994]. Similarly, transgenic mice that overexpress *Dax1* display gonadal abnormalities in male mice, with the severity depending on the mouse strain [Swain et al.,

1998]. In contrast to humans, XY *Dax1* null mice do show defects in testis development [Yu et al., 1998]. *Dax1* is expressed during the critical time window of early gonadal sex determination. After *Sry* expression ceases, *Dax1* is downregulated in the developing testis but maintains its expression levels in the ovary [Swain et al., 1998]. *DAX1* has been shown by in vitro experiments to interact with *SF1* thereby repressing the synergistic action of *SF1* and *WT1* on the *Amh* promoter [Nachtigal et al., 1998]. *Dax1* null XX female mice develop functional ovaries, suggesting that *DAX1* is not an ovarian-determining gene but, if duplicated, acts antagonistically to *SRY* as an anti-testis gene. Klinefelter's syndrome (XXY) individuals are male, not sex-reversed females and this is likely due to X chromosome inactivation of *DAX1*. It has been suggested that *DAX1* might function within an activity window [Ludbrook and Harley, 2004]. If *DAX1* is present in a certain concentration, it can promote proper testis development. On the other hand, too little or too much *DAX1* activity leads to misregulated testis development.

ATRX is a member of the SWI/SNF family of chromatin-remodeling proteins. Mutations and deletions of the *ATRX* gene cause the alpha-thalassemia/mental retardation syndrome, X-linked [Gibbons et al., 1995]. XY patients additionally show varying degrees of 46,XY DSD, implicating alpha-thalassemia/mental retardation syndrome in testis development [Reardon et al., 1995]. However, the range of observed genital phenotypes, from small testes to ambiguous external genitalia, and the lack of complete male-to-female sex reversal suggest *ATRX* plays a role in sex (testis) differentiation rather than in the earlier gonadal sex (testis) determination.

GATA4 is one of 6 members of the evolutionarily conserved *GATA* family of transcription factors. *GATA* factors contain 2 zinc finger domains that have been shown to be critical for DNA recognition and binding, as well as for protein-protein binding with other transcription cofactors. *GATA4* has been shown to be expressed in somatic cells in the gonads of both sexes before and during the time of sex determination [Viger et al., 1998]. In the mouse, *Gata4* expression becomes testis-specific at E13.5, and its expression is maintained in Sertoli cells after birth. *GATA4* usually interacts with other transcription factors to regulate the expression of its target genes. Two well-known partners are *NR5A1/SF1* and *FOG2/ZFPM2* (friend of *GATA-2*/zinc finger protein, *FOG* family member 2). Together, they have been shown to regulate the sex determination and differentiation genes *SRY*, *SOX9* and *AMH*, as well as *STAR* (steroidogenic acute regulatory protein), *CYP19A1* (cytochrome P450, family 19, sub-

family A, polypeptide 1) and *HSD3B2* (hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2). *Gata4*^{-/-} mice die before the development of bipotential gonads; however, *Gata4*^{ki} mice carrying a mutation in the N-terminal zinc finger domain, which prevents the interaction with FOG2, exhibit severe anomalies of testis development [Bouma et al., 2007]. Most described mutations in human *GATA4* have been linked to congenital heart defects (CHD) only. However, a recent study reported a familial case of a heterozygous mutation in *GATA4* with three 46,XY DSD and two 46,XX CHD cases [Lourenço et al., 2011]. In another study, a 35-kb deletion downstream of *GATA4* was discovered in a 46,XY complete gonadal dysgenesis patient without CHD [White et al., 2011].

FOG2 was shown to repress *GATA4*-dependent activation of *Amh* expression in rat primary Sertoli cells. *Fog2*^{-/-} mice show a block of gonadal development and fail to develop testes. However, it is unclear whether FOG2 acts as an activator or repressor (or both) of gonadal gene expression. So far, only one translocation including *FOG2* (leading to a truncated FOG2 protein) in a boy with hypergonadotropic hypogonadism and CHD has been described [Finelli et al., 2007].

Two members of the mitogen-activated protein kinase (MAPK) signaling pathway, *MAP3K1* and *Map3k4*, have recently been added to the growing list of genes involved in testis development. Linkage analysis of 2 affected families and gene sequencing of several individuals with 46,XY DSD revealed mutations in the *MAP3K1* gene as the likely cause for the condition [Pearlman et al., 2010]. In mice, *Map3k1* appears to play only a minor role in testis development as *Map3k1* null mice do not show gonadal abnormalities [Warr et al., 2011]. However, using a forward genetic screen in mice, a mutation in the *Map3k4* gene responsible for male-to-female sex reversal was discovered. Expression analysis of *Map3k4* null XY gonads revealed a dramatic reduction of *Sry* and *Sox9* expression [Bogani et al., 2009]. A recent report proposed a model of interaction to explain this observation [Warr et al., 2012]. According to this model, GADD45G (growth arrest and DNA-damage-inducible 45 gamma) activates MAP3K4, which in turn leads to the activation of p38 MAP kinases (MAPK11/MAPK14) and subsequently *GATA4*, an important transcriptional regulator of several testis genes, including *Sry* and *Sox9*. Taken together, these findings point to an important function of the MAPK signaling pathway in testis development.

Ovarian Development

Until very recently, the pathway leading to ovarian development was considered to be a passive or 'default' pathway, and little was known about the underlying molecular mechanisms leading to functional ovaries. However, in recent years, several important players of ovarian development have been identified, and it has become clear that the ovarian pathway is not passive. Furthermore, it has become apparent that components of both male and female pathways antagonize each other to ensure proper development of either testes or ovaries.

CTNNB1/beta catenin (catenin, beta 1) is a key component of the WNT (wingless-type MMTV integration site family) signaling pathway and a central player in ovarian development. It acts as a transcriptional regulator and, if activated by WNT ligand binding, executes its function by binding to TCF/LEF (transcription factor/lymphoid enhancer-binding factor 1) transcription factors that in turn activate the expression of their target genes. In the absence of beta-catenin, TCF/LEF acts as a transcriptional repressor by recruiting Groucho proteins [Eastman and Grosschedl, 1999]. In mice, stabilization by phosphorylation of beta-catenin in XY gonads has been shown to promote ovarian development and suppress testis development, leading to XY sex reversal [Maatouk et al., 2008]. The role of beta-catenin in human gonadal development has not so far been established. However, 2 signaling molecules that activate beta-catenin, *WNT4* and *RSPO1* (R-spondin 1) (see below), are upregulated in the female gonad and – in contrast to beta-catenin – have been shown to cause DSDs.

The *WNT* gene family encodes locally acting signaling molecules that are involved in a wide range of developmental processes. *WNT4* is its most important member in gonadal development. In the mouse, *Wnt4* is initially expressed in both sexes. While its expression levels are increasing in the ovary during gonad differentiation, it is downregulated in the testis from 12.5 days post coitum. In humans, homozygous null mutations were shown to cause 46,XX testicular DSD [Mandel et al., 2008]. Interestingly, *Wnt4* knockout XX mice show a milder phenotype with only partial sex reversal. These mice lacked Müllerian ducts and failed to maintain oocytes [Vainio et al., 1999]. Furthermore, overexpression of *Wnt4* in XY mice results in disruption of testicular vasculature and inhibition of testosterone production by suppressing Leydig cell development, but does not lead to sex reversal [Jordan et al., 2003]. Taken together, these findings show that *WNT4* alone is not able to override the male devel-

Fig. 1. Overview of the genes and their interactions in gonadal sex determination. Arrows indicate activation of a downstream target. Lines ending in bars indicate repression of a downstream target. The interactions shown are not necessarily direct. KDM3A was not included as its effects are epigenetic. Refer to the text for more detailed information.

opmental program, and hence, WNT4 on its own cannot be the sole ovarian determinant.

Homozygous null mutations in *RSPO1*, a member of the R-spondin family, were shown to cause 46,XX testicular DSD in humans [Parma et al., 2006]. This was the first report of a single gene mutation leading to female-to-male sex reversal. *RSPO1* is thought to activate and then synergize with WNT4 in embryonic ovaries to stabilize beta-catenin and thereby allowing the female pathway to proceed [Chassot et al., 2008]. The conservation and expression pattern of *RSPO1* in other vertebrates points to an important role in female sex determination. In mice however, it appears to act together with other factors, as *Rspo1* null mice display only partial sex reversal [Tomizuka et al., 2008]. Interestingly, a human patient with a duplication of chromosome 1p, containing both *WNT4* and *RSPO1*, had a female phenotype with 46,XY DSD [Jordan et al., 2001]. Presumably, the duplication has affected expression of WNT4 and *RSPO1*.

FOXL2 is a member of the evolutionarily conserved forkhead box (FOX) superfamily of transcription factors. In vertebrates, *FOXL2* is one of the earliest markers of ovarian development. In goats, a deletion affecting the expression of *FOXL2* has been shown to cause female-to-male sex reversal [Pailhoux et al., 2001]. In humans, loss-of-function mutations of *FOXL2* cause blepharophimosis, ptosis and epicanthus inversus syndrome, a genetic disorder leading to complex eyelid malformation and

other craniofacial abnormalities [Crisponi et al., 2001]. This syndrome can be associated with premature ovarian insufficiency, suggesting a role for *FOXL2* in follicle maintenance during adult life [Harris et al., 2002]. However, no link between *FOXL2* and human DSD has been established so far. While XX *Foxl2* null mice show only masculinization of the supporting cells in the gonad, double knockouts of both *Foxl2* and *Wnt4* lead to female-to-male sex reversal and development of male germ cells [Ottolenghi et al., 2007]. Interestingly, *FOXL2* also plays an important role in maintaining functional ovaries in adult mice (see below). The exact relationship between *RSPO1*/WNT4 and *FOXL2* in coordinating ovarian development remains unclear.

Testes and Ovaries Actively Suppress Opposing Pathways to Ensure Their Proper Development and Maintenance

Components of both male and female sex differentiation pathways have been shown to act antagonistically (fig. 1). During embryonic development the main players appear to be *SOX9* and beta-catenin. In males, *SRY*-induced increased expression of *SOX9* limits the expression of female-specific genes. The positive feedback loop of *SOX9* and *FGF9* actively suppresses the activation of WNT4 to tip the balance towards testis development

[Kim et al., 2006]. By contrast, beta-catenin, stabilized and activated by WNT4 and RSPO1, limits the expression of SOX9 by inhibiting the SOX9/FGF9 feedback loop to ensure the development of ovaries in females [Maatouk et al., 2008]. Recently, an additional player has been added to the opposing testicular and ovarian pathways. Loke et al. [2013] were able to show that splice-site and mis-sense mutations of MAP3K1 tilt the balance from male (FGF9/SOX9) to the female (WNT4/beta-catenin) pathway.

The battle of the sexes is not completely resolved during embryogenesis but continues into adulthood long after gonadal sex determination and differentiation are completed. Female *Cyp19a1* knockout mice have been shown to contain Sertoli-like cells in their ovaries. However, estradiol administration reverses the transdifferentiation of granulosa cells into Sertoli-like cells in female *Cyp19a1* knockout mice [Britt et al., 2004]. Further evidence for this life-long battle came from 2 recent remarkable publications that analyzed the role of FOXL2 and DMRT1 in adult ovaries and testes, respectively. In mice, when *Foxl2* is conditionally knocked out in granulosa cells of fully differentiated adult ovaries, a striking event occurs. The granulosa cells of the ovary transdifferentiate into Sertoli-like cells, theca cells become Leydig cells and, instead of the typical follicular structure of an ovary, the gonads resemble a testis in appearance with seminiferous tubules [Uhlenhaut et al., 2009]. This clearly indicates that FOXL2 is required for maintenance of adult ovaries and suppression of the testicular pathway throughout adult life. On the other hand, when *Dmrt1* is conditionally knocked out in postnatal Sertoli cells, *Foxl2* becomes activated, Sertoli cells transdifferentiate into granulosa cells, the gonads start to produce estrogen, and germ cells become feminized [Matson et al., 2011]. This clearly shows that DMRT1 is necessary to maintain the adult testis. Surprisingly, both testes and ovaries appear labile even after birth and apparently require constitutive gene expression to suppress the opposing pathway from being activated.

Novel Strategies to Identify Missing Components of Sex Differentiation Pathways

As most cases of DSD lead to infertility, many mutations affecting sex determination and development are spontaneous, and familial cases are not very common. Recent analyses of DSD patients using SNP arrays to identify genome-wide duplications and deletions have

identified many rearrangements in intergenic regions, some of them in proximity to known sex genes [White et al., 2011]. This strongly suggests that many cases of these disorders might be due to disruptions of regulatory regions of sex determination and differentiation genes. Even ectopic expression of nongonadal genes can lead to DSD. In a recent study, genomic rearrangements surrounding or including SOX3 were found in several 46,XX testicular DSD patients [Sutton et al., 2011]. The X-linked SOX3 gene – considered to be the ancestor of the Y-linked SRY – is normally not expressed in gonads at any developmental stage. It is therefore likely that the rearrangements led to ectopic expression of SOX3 in the developing gonads of these patients during embryonic development, where it was able to substitute for SRY and drive the testicular pathway.

Despite recent technical advancements, it is still very laborious and difficult to identify the exact location and unravel the function of regulatory regions. Chromatin immunoprecipitation followed by deep sequencing using antibodies for transcription factors involved in gonadal development and differentiation (e.g. SOX9 and FOXL2) will help to identify important targets of these transcription factors and likely lead to the discovery of hitherto unknown gonadal genes. The same approach using antibodies specific for certain chromatin marks (e.g. H3K4me1, H3K27Ac) will increase our understanding of the regulation of gonadal genes by identifying their enhancers and promoters. Due to the nature of SNP arrays and their limitation in resolution, rearrangements smaller than 5–10 kb cannot be detected by these arrays. However, due to technological advances and the rapidly falling costs, it will soon be feasible to routinely perform whole exome or even whole genome sequencing of DSD patients to detect genomic rearrangements and mutations down to the single nucleotide level. The next big challenge will be to make sense of the massive amounts of data generated by these new technologies.

Concluding Remarks

Since the discovery of SRY over 2 decades ago, there has been a dramatic increase in our understanding of the molecular mechanisms and genetic interactions involved in human sex determination and differentiation. The identification and characterization of several key genes has helped to elucidate the complexity inherent in the development of functional testes and ovaries. However, many questions concerning sexual development still re-

main unanswered. The majority of human DSD cases still cannot be explained. While environmental factors might play a role, it is likely that more components of this complex genetic network await discovery. Rapidly evolving technology such as massively parallel sequencing of DSD patients will enable us to identify more genes that are

components of the testis and ovarian pathways. This will not only provide insights into human gonadal sex determination and sexual differentiation but also will assist with the diagnosis and subsequent clinical management of patients with DSD.

References

- Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL: A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. *Nat Genet* 22: 125–126 (1999).
- Arango NA, Lovell-Badge R, Behringer RR: Targeted mutagenesis of the endogenous mouse *Mis* gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. *Cell* 99:409–419 (1999).
- Bagheri-Fam S, Sim H, Bernard P, Jayakody I, Taketo MM, et al: Loss of *Fgfr2* leads to partial XY sex reversal. *Dev Biol* 314:71–83 (2008).
- Barbaux S, Niaudet P, Gubler MC, Grünfeld JP, Jaubert F, et al: Donor splice-site mutations in *WT1* are responsible for Frasier syndrome. *Nat Genet* 17:467–470 (1997).
- Barriouneuve F, Bagheri-Fam S, Klattig J, Kist R, Taketo MM, et al: Homozygous inactivation of *Sox9* causes complete XY sex reversal in mice. *Biol Reprod* 74:195–201 (2006).
- Baskin LS, Himes K, Colborn T: Hypospadias and endocrine disruption: is there a connection? *Environ Health Perspect* 109:1175–1183 (2001).
- Baumstark A, Barbi G, Djalali M, Geerkens C, Mitulla B, et al: Xp-duplications with and without sex reversal. *Hum Genet* 97:79–86 (1996).
- Beleza-Meireles A, Lundberg F, Lagerstedt K, Zhou X, Omrani D, et al: *FGFR2*, *FGF8*, *FGF10* and *BMP7* as candidate genes for hypospadias. *Eur J Hum Genet* 15:405–410 (2007).
- Benko S, Gordon CT, Mallet D, Sreenivasan R, Thauvin-Robinet C, et al: Disruption of a long distance regulatory region upstream of *SOX9* in isolated disorders of sex development. *J Med Genet* 48:825–830 (2011).
- Biason-Lauber A, Konrad D, Meyer M, DeBeaufort C, Schoenle EJ: Ovaries and female phenotype in a girl with 46,XY karyotype and mutations in the *CBX2* gene. *Am J Hum Genet* 84:658–663 (2009).
- Bitgood MJ, Shen L, McMahon AP: Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr Biol* 6:298–304 (1996).
- Bogani D, Siggers P, Brixey R, Warr N, Beddow S, et al: Loss of mitogen-activated protein kinase kinase 4 (MAP3K4) reveals a requirement for MAPK signalling in mouse sex determination. *PLoS Biol* 7:e1000196 (2009).
- Bouma GJ, Washburn LL, Albrecht KH, Eicher EM: Correct dosage of *Fog2* and *Gata4* transcription factors is critical for fetal testis development in mice. *Proc Natl Acad Sci USA* 104:14994–14999 (2007).
- Britt KL, Stanton PG, Misso M, Simpson ER, Findlay JK: The effects of estrogen on the expression of genes underlying the differentiation of somatic cells in the murine gonad. *Endocrinology* 145:3950–3960 (2004).
- Canto P, Söderlund D, Reyes E, Méndez JP: Mutations in the desert hedgehog (*DHH*) gene in patients with 46,XY complete pure gonadal dysgenesis. *J Clin Endocrinol Metab* 89:4480–4483 (2004).
- Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, et al: Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45:685–698 (1986).
- Chassot A-A, Ranc F, Gregoire EP, Roepers-Gajdian HL, Taketo MM, et al: Activation of beta-catenin signaling by *Rspo1* controls differentiation of the mammalian ovary. *Hum Mol Genet* 17:1264–1277 (2008).
- Chiang H-S, Wu Y-N, Wu C-C, Hwang J-L: Cytogenic and molecular analyses of 46,XX male syndrome with clinical comparison to other groups with testicular azoospermia of genetic origin. *J Formos Med Assoc* 112:72–78 (2013).
- Clépet C, Schafer AJ, Sinclair AH, Palmer MS, Lovell-Badge R, Goodfellow PN: The human *SRY* transcript. *Hum Mol Genet* 2:2007–2012 (1993).
- Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM: Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* 104:875–889 (2001).
- Cox JJ, Willatt L, Homfray T, Woods CG: A *SOX9* duplication and familial 46,XX developmental testicular disorder. *N Engl J Med* 364:91–93 (2011).
- Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, et al: The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat Genet* 27:159–166 (2001).
- Eastman Q, Grosschedl R: Regulation of Lef-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* 11:233–240 (1999).
- Finelli P, Pincelli AI, Russo S, Bonati MT, Recalcati MP, et al: Disruption of friend of GATA 2 gene (*FOG-2*) by a de novo t(8;10) chromosomal translocation is associated with heart defects and gonadal dysgenesis. *Clin Genet* 71:195–204 (2007).
- Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, et al: Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. *Nature* 372:525–530 (1994).
- Gibbons RJ, Picketts DJ, Villard L, Higgs DR: Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). *Cell* 80:837–845 (1995).
- Giese K, Cox J, Grosschedl R: The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell* 69:185–195 (1992).
- Gordon CT, Tan TY, Benko S, FitzPatrick D, Lyonnet S, Farlie PG: Long-range regulation at the *SOX9* locus in development and disease. *J Med Genet* 46:649–656 (2009).
- Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, et al: Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. *Cell* 106:319–329 (2001).
- Hanley NA, Hagan DM, Clement-Jones M, Ball SG, Strachan T, et al: *SRY*, *SOX9*, and *DAX1* expression patterns during human sex determination and gonadal development. *Mech Dev* 91:403–407 (2000).
- Harris SE, Chand AL, Winship IM, Gersak K, Aitomäki K, Shelling AN: Identification of novel mutations in *FOXL2* associated with premature ovarian failure. *Mol Hum Reprod* 8:729–733 (2002).
- Hohenstein P, Hastie ND: The many facets of the Wilms' tumour gene, *WT1*. *Hum Mol Genet* 15 Spec No 2:R196–R201 (2006).
- Huang B, Wang S, Ning Y, Lamb AN, Bartley J: Autosomal XX sex reversal caused by duplication of *SOX9*. *Am J Med Genet* 87:349–353 (1999).
- Hughes IA, Houk C, Ahmed SF, Lee PA, LWPES Consensus Group, ESPE Consensus Group: Consensus statement on management of intersex disorders. *J Pediatr Urology* 2:148–162 (2006).

- Jordan BK, Mohammed M, Ching ST, Délot E, Chen XN, et al: Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans. *Am J Hum Genet* 68:1102–1109 (2001).
- Jordan BK, Shen JH-C, Olosa R, Ingraham HA, Vilain E: Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. *Proc Natl Acad Sci USA* 100:10866–10871 (2003).
- Kanai Y, Koopman P: Structural and functional characterization of the mouse *Sox9* promoter: implications for campomelic dysplasia. *Hum Mol Genet* 8:691–696 (1999).
- Katoh-Fukui Y, Tsuchiya R, Shiroishi T, Nakahara Y, Hashimoto N, et al: Male-to-female sex reversal in *M33* mutant mice. *Nature* 393:688–692 (1998).
- Katoh-Fukui Y, Miyabayashi K, Komatsu T, Owaki A, Baba T, et al: *Cbx2*, a polycomb group gene, is required for *Sry* gene expression in mice. *Endocrinology* 153:913–924 (2012).
- Kim Y, Kobayashi A, Sekido R, DiNapoli L, Brennan J, et al: *Fgf9* and *Wnt4* act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol* 4:e187 (2006).
- Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R: Male development of chromosomally female mice transgenic for *Sry*. *Nature* 351:117–121 (1991).
- Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, et al: WT-1 is required for early kidney development. *Cell* 74:679–691 (1993).
- Kuroki S, Matoba S, Akiyoshi M, Matsumura Y, Miyachi H, et al: Epigenetic regulation of mouse sex determination by the histone demethylase *Jmjd1a*. *Science* 341:1106–1109 (2013).
- Loke J, Pearlman A, Radi O, Zuffardi O, Giussani U, et al: Mutations in *MAP3K1* tilt the balance from *SOX9*/*FGF9* to *WNT*/*beta*-catenin signaling. *Hum Mol Genet* (2013), E-pub ahead of print.
- Lourenço D, Brauner R, Lin L, De Perdigo A, Weryha G, et al: Mutations in *NR5A1* associated with ovarian insufficiency. *N Engl J Med* 360:1200–1210 (2009).
- Lourenço D, Brauner R, Rybczynska M, Nihoul-Fékété C, McElreavey K, Bashamboo A: Loss-of-function mutation in *GATA4* causes anomalies of human testicular development. *Proc Natl Acad Sci USA* 108:1597–1602 (2011).
- Ludbrook LM, Harley VR: Sex determination: a 'window' of DAX1 activity. *Trends Endocrinol Metab* 15:116–121 (2004).
- Luo X, Ikeda Y, Parker KL: A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* 77:481–490 (1994).
- Maatouk DM, DiNapoli L, Alvers A, Parker KL, Taketo MM, Capel B: Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. *Hum Mol Genet* 17:2949–2955 (2008).
- Mandel H, Shemer R, Borochowitz ZU, Okopnik M, Knopf C, et al: SERKAL syndrome: an autosomal-recessive disorder caused by a loss-of-function mutation in *WNT4*. *Am J Hum Genet* 82:39–47 (2008).
- Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D: DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476:101–104 (2011).
- Muscattelli F, Strom TM, Walker AP, Zanaria E, Récan D, et al: Mutations in the *DAX-1* gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature* 372:672–676 (1994).
- Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA: Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* 93:445–454 (1998).
- Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, et al: Loss of *Wnt4* and *Foxl2* leads to female-to-male sex reversal extending to germ cells. *Hum Mol Genet* 16:2795–2804 (2007).
- Pailhoux E, Vigier B, Chaffaux S, Servel N, Taourit S, et al: A 11.7-kb deletion triggers intersexuality and polledness in goats. *Nat Genet* 29:453–458 (2001).
- Parma P, Radi O, Vidal V, Chaboissier M-C, Delambra E, et al: R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet* 38:1304–1309 (2006).
- Pearlman A, Loke J, Le Caignec C, White S, Chin L, et al: Mutations in *MAP3K1* cause 46,XY disorders of sex development and implicate a common signal transduction pathway in human testis determination. *Am J Hum Genet* 87:898–904 (2010).
- Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, et al: Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* 67:437–447 (1991).
- Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, et al: Evidence for evolutionary conservation of sex-determining genes. *Nature* 391:691–695 (1998).
- Raymond CS, Parker ED, Kettlewell JR, Brown LG, Page DC, et al: A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Hum Mol Genet* 8:989–996 (1999).
- Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D: *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14:2587–2595 (2000).
- Reardon W, Gibbons RJ, Winter RM, Baraitser M: Male pseudohermaphroditism in sibs with the alpha-thalassemia/mental retardation (ATR-X) syndrome. *Am J Med Genet* 55:285–287 (1995).
- Sanlaville D, Vialard F, Thépot F, Vue-Droy L, Ardalán A, et al: Functional disomy of Xp including duplication of *DAX1* gene with sex reversal due to t(X;Y)(p21.2;p11.3). *Am J Med Genet* 128A:325–330 (2004).
- Satoh M: The histogenesis of the gonad in rat embryos. *J Anat* 143:17–37 (1985).
- Schmahl J, Kim Y, Colvin JS, Ornitz DM, Capel B: *Fgf9* induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. *Development* 131:3627–3636 (2004).
- Sekido R, Lovell-Badge R: Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer. *Nature* 453:930–934 (2008).
- Sekido R, Bar I, Narváez V, Penny G, Lovell-Badge R: SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. *Dev Biol* 274:271–279 (2004).
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, et al: A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346:240–244 (1990).
- Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, et al: The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature* 461:267–271 (2009).
- Sutton E, Hughes J, White S, Sekido R, Tan J, et al: Identification of *SOX3* as an XX male sex reversal gene in mice and humans. *J Clin Invest* 121:328–341 (2011).
- Swain A, Narvaez V, Burgoyne P, Camerino G, Lovell-Badge R: *Dax1* antagonizes *Sry* action in mammalian sex determination. *Nature* 391:761–767 (1998).
- Tannour-Louet M, Han S, Corbett ST, Louet J-F, Yatsenko S, et al: Identification of de novo copy number variants associated with human disorders of sexual development. *PLoS One* 5:e15392 (2010).
- Tevosian SG, Albrecht KH, Crispino JD, Fujiwara Y, Eicher EM, Orkin SH: Gonadal differentiation, sex determination and normal *Sry* expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development* 129:4627–4634 (2002).
- Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, et al: R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum Mol Genet* 17:1278–1291 (2008).
- Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, et al: Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139:1130–1142 (2009).
- Umehara F, Tate G, Itoh K, Yamaguchi N, Douchi T, et al: A novel mutation of desert hedgehog in a patient with 46,XY partial gonadal dysgenesis accompanied by minifascicular neuropathy. *Am J Hum Genet* 67:1302–1305 (2000).

- Vainio S, Heikkilä M, Kispert A, Chin N, McMahon AP: Female development in mammals is regulated by Wnt-4 signalling. *Nature* 397: 405–409 (1999).
- Val P, Lefrançois-Martinez A-M, Veyssière G, Martinez A: SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl Recept* 1:8 (2003).
- Vetro A, Ciccone R, Giorda R, Patricelli MG, Della Mina E, et al: XX males SRY negative: a confirmed cause of infertility. *J Med Genet* 48: 710–712 (2011).
- Vidal VP, Chaboissier MC, de Rooij DG, Schedl A: *Sox9* induces testis development in XX transgenic mice. *Nat Genet* 28:216–217 (2001).
- Viger RS, Mertineit C, Trasler JM, Nemer M: Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter. *Development* 125:2665–2675 (1998).
- Wagner T, Wirth J, Meyer J, Zabel B, Held M, et al: Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene *SOX9*. *Cell* 79: 1111–1120 (1994).
- Warr N, Bogani D, Siggers P, Brixey R, Tateossian H, et al: Minor abnormalities of testis development in mice lacking the gene encoding the MAPK signalling component, MAP3K1. *PLoS One* 6:e19572 (2011).
- Warr N, Carre G-A, Siggers P, Faleato JV, Brixey R, et al: *Gadd45gamma* and *Map3k4* interactions regulate mouse testis determination via p38 MAPK-mediated control of *Sry* expression. *Dev Cell* 23:1020–1031 (2012).
- White S, Ohnesorg T, Notini A, Roeszler K, Hewitt J, et al: Copy number variation in patients with disorders of sex development due to 46,XY gonadal dysgenesis. *PLoS One* 6:e17793 (2011).
- Wilhelm D, Englert C: The Wilms tumor suppressor WT1 regulates early gonad development by activation of *Sfl*. *Genes Dev* 16: 1839–1851 (2002).
- Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, et al: The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. *Nat Genet* 9:15–20 (1995).
- Yu RN, Ito M, Saunders TL, Camper SA, Jameson JL: Role of *Ahch* in gonadal development and gametogenesis. *Nat Genet* 20:353–357 (1998).