

Disorders of Sex Development (DSDs): An Update

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Context: Disorders of sex development (DSDs) may arise from genetic defects in testis or ovary determination. Current analytical technologies and improved understanding of major regulatory pathways have cast new insight into the genetic basis for these disorders.

Evidence Acquisition: A PubMed search was performed for the years 2011–13 using the terms “disorder of sex development,” “gonadal dysgenesis,” “ovarian dysgenesis,” “array CGH,” and “whole exome sequencing.” Only articles from peer-reviewed journals were included.

Evidence Synthesis: Key themes that emerged included aberrant regulation of SOX9 via the hTES promoter in 46,XY gonadal DSDs, the role of the MAPK pathway in normal and aberrant gonadal development, and the role of new technologies in identification of gonadal DSDs.

Conclusions: With the advent of the robust new technologies of array comparative genomic hybridization and genomic sequencing in recent years, many new sex-determining genes have been identified. These genes have been organized into ovarian- and testicular-determining pathways that can block each other’s activities. Identification of a mutation in a sex-determining gene in an individual affected with a DSD may warrant more extensive investigation for other phenotypic effects as well as genetic testing of other family members. (*J Clin Endocrinol Metab* 99: 1503–1509, 2014)

Disorders of sex development (DSDs) are congenital conditions in which development of the chromosomal, gonadal, or anatomic sex is atypical and may affect up to 1:1000 individuals in the population (1–3). Some of these disorders primarily affect the gonads, including 46,XX testicular DSD (formerly called “XX maleness”) and 46,XY DSD with partial or complete gonadal dysgenesis. These conditions may be identified at different times of the life cycle—in fetuses or newborns with ambiguous external genitalia, dysgenetic gonads, and internal genitalia that are discordant for sex chromosome constitution. Alternatively, they may be diagnosed later in individuals with delayed puberty, unanticipated virilization or gynecomastia, infertility, or gonadal tumors. The risk of gonadal tumors may be as high as 40% in individuals with dysgenetic gonads (4). Indeed, dysgenetic and undescended testes are the major risk factors for testicular

cancer, the most common malignancy for men between the ages of 15 and 35 years. Sometimes the gonadal DSD may be a feature of a genetic syndrome, such as campomelic dysplasia or adrenal hypoplasia congenital, or a newly characterized condition, thereby demonstrating the multiple phenotypic effects of mutations. Diagnosis relies on clinical findings, hormonal analysis, gonadal histology, chromosome analysis, and genetic testing.

Whereas general health and cognitive ability of individuals with DSDs are usually not impaired, the diagnosis can be challenging for the proband and his/her parents (5). Individuals may be assigned a gender of rearing that is discordant for gender identity. Sometimes this leads to gender dysphoria later in life and may require a reassignment. The proband may require gender-corrective surgery of external and internal genitalia, removal of part or whole of dysgenetic gonads or ovotestes and possible relocation

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

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Received October 5, 2013. Accepted February 27, 2014.

First Published Online April 18, 2014

Abbreviations: CGH, comparative genomic hybridization; DSD, disorders of sex development; FVA, Functional variant assay; hTES, human testis-specific enhancer of Sox9; mTES, mouse testis-specific enhancer of Sox9.

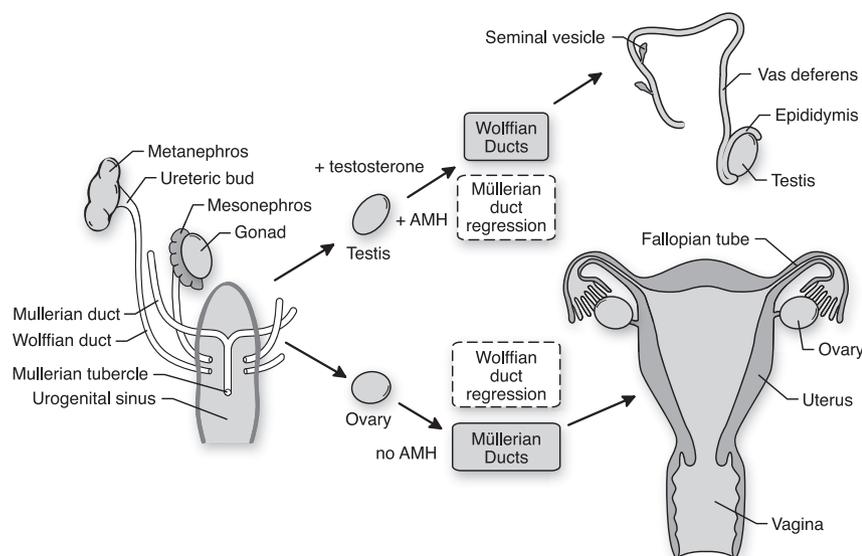


Figure 1. Development of internal genitalia from common genital structures. Ordinarily, the *SRY* gene on the Y chromosome would trigger testes and subsequent male internal genital development from production of the hormones, T and anti-Müllerian hormone (AMH). In 46,XX testicular DSD, testis determination can be triggered by *SRY* or *SOX9* translocation, *SOX3* or *SOX9* duplication, or *RSPO1* loss of function. In 46,XY gonadal dysgenesis, testis development does not occur or is incomplete, and internal female genitalia will develop. This will be triggered by *SRY*, *NR5A1*, *DHH*, or testis-determining gene loss of function mutations, *DAX1* or *WNT4* duplication, or *MAP3K1* gain of function mutations.

of gonads, and hormonal replacement starting in infancy or adolescence and extending into adulthood. Moreover, the proband may not be the only affected member of the family (6).

Evidence for sex-determining transcription factor and signaling molecule genes emerged initially from identification of chromosomal abnormalities and subsequently from identification of mutations in genes in individuals with gonadal DSDs. Among the genes identified were *SRY* (7), *SOX9* (8, 9), *NR5A1* (10), *WT1* (11, 12), *DAX1* (13), *WNT4* (14), *CBX2* (15), *DMRT1* (16), and *GATA4* (17). (Note that gene names are indicated in italics.) One theme of this work is that gene dosage and resulting level of gene expression may be critical for testis determination. Expression of a single copy of the *SOX9*, *SF1*, and *WT1* genes in 46,XY individuals can lead to gonadal dysgenesis (10, 18–21). Duplication of the *DAX1* and *WNT4* genes in 46,XY individuals can also lead to gonadal dysgenesis, whereas duplication of the *SOX9* or *SOX3* genes can lead to 46,XX testicular DSD (14, 22–25).

These observations fit a genetic model that explains the pathogenesis of gonadal DSDs (Figure 1). Expression of a gene on the Y chromosome initiates a genetic cascade that causes the undifferentiated gonad to develop as a testis (26). In turn, hormones secreted by the testis cause the Wolffian ducts to differentiate as seminal vesicles, vas deferens, and epididymis and cause the Müllerian ducts to regress. In the absence of a Y chromosome and expression of this gene, a testis does not develop (27), the Wolffian

ducts regress, and the Müllerian ducts develop as fallopian tubes, uterus, and upper third of the vagina. If the genetic pathway of gonadal development is faulty, so that gene functions are lost or overridden, DSDs result (4).

Here, I review recent developments in the genetics of gonadal DSDs. This work has shown that mutations in *SRY*, *NR5A1*, and *SOX9* all coalesce in their effects by regulating the expression of the *SOX9* gene through its human testis-specific enhancer of *Sox9* (hTES) promoter. Specific mutations in the signal transduction gene, *MAP3K1*, cause 46,XY partial or complete gonadal dysgenesis by altering the activities of signaling molecules and transcription factors, including β -catenin and *SOX9*. New genes have been identified from the appli-

cation of contemporary technologies, including array comparative genomic hybridization (CGH) and genomic sequencing. Together, these recent developments provide a more complete view of the genetic control of sex determination and its disorders. When placed in an evolutionary context, certain key features of the pathways are conserved despite a variety of sex-determining mechanisms.

Aberrant Regulation of *SOX9* via the hTES Promoter in 46,XY Gonadal DSDs

When mutated, the human *SOX9* gene causes campomelic dysplasia, a condition of long bone bowing in the legs and sometimes in the arms, and frequently 46,XY partial or complete gonadal dysgenesis (8, 9). Conversely, duplications and translocations of the *SOX9* gene or upstream enhancer region, presumably resulting in overexpression of the gene product, result in 46,XX testicular DSD (25, 28–30). These effects have been mimicked in the mouse. Overexpression of a *Sox9* transgene in XX mice results in testis development in the absence of *Sry*, whereas knock-out of this gene results in the absence of testis development in XY mice (31–33). These observations, coupled with the finding that enhanced *Sox9* expression occurs in Sertoli cell precursors just after the onset of *Sry* expression, led to the proposal that *Sox9* could be directly regulated by *Sry*. The molecular basis for this regulation

in humans, including the roles of mutations in the *SRY*, *SOX9*, and *NR5A1* genes in disrupting this regulation, has been studied.

In mice, *Sry* interacts cooperatively with *Nr5a1* at the mouse testis-specific enhancer of *Sox9* (mTES) in the *Sox9* gene to up-regulate expression of this gene (34). After *Sry* expression has ceased, *Sox9* itself interacts cooperatively with *Nr5a1* at mTES to maintain its own expression. A similar mechanism operates at the hTES promoter to regulate expression of *SOX9* in the human embryonal carcinoma cell line (NT2/D1) (35). Overexpression of transfected *SRY* in NT2/D1 cells increases endogenous *SOX9* expression. This up-regulation is associated with *SRY* localization to actively transcribed chromatin and is augmented by cotransfection with *NR5A1*. Similar augmentation is observed for cotransfection of *SOX9* and *NR5A1*.

SRY, *NR5A1*, and *SOX9* mutations observed in individuals with 46,XY DSD partial or complete gonadal dysgenesis have a reduced ability to activate hTES. The mutations in *SRY*, *NR5A1*, and *SOX9* varied in their transactivation of *SOX9* depending on whether they acted as highly penetrant dominant alleles or hypomorphic alleles. Examples of hypomorphic alleles are familial mutations in *SRY* that are transmitted by nonmosaic fertile fathers, but cause 46,XY gonadal dysgenesis in offspring and recessive mutations in *NR5A1* that cause 46,XY gonadal dysgenesis only when two alleles have been inherited (6, 36). The highly penetrant, dominant mutations in these genes demonstrated the greatest effect on transactivation via hTES, whereas the hypomorphic alleles had a lesser effect. Mutations in the known TES region have not been identified in human 46,XY DSDs (37). This overall pattern of regulation in NT2/D1 cells models the events in pre-Sertoli cells and demonstrates that *SOX9* is a central hub gene in testis determination that can be subject to additional regulation. The up-regulation and sexually dimorphic expression pattern of *SOX9* are consistent across all vertebrate species, regardless of the switch that controls sex determination—*SRY* in heterogametic XY mammals (19, 21), ZZ heterogametic birds and reptiles (38), and temperature-sensitive egg incubation in turtles and crocodiles (39, 40).

Role of the MAPK Pathway in Normal and Aberrant Gonadal Development

Based on the analysis of familial and sporadic cases of 46,XY partial and complete gonadal dysgenesis, mutations in the *MAP3K1* gene were shown to be a common, if not the most common, cause (13–18% of cases) (41–

43). In familial cases, these mutations demonstrated coinheritance with the phenotype and significant LOD scores by linkage analysis (>5 for multipoint linkage analysis in the first family studied) (41). The mutations occurred at well-conserved sites in exons 2, 3, 13, and 14 of this 20 exon gene and have the characteristic of being in-frame alterations, either nonconservative single-nucleotide variants or familial splice acceptor site variants that resulted in in-frame insertions or deletions. None of these mutations resulted in diminished or unstable MAP3K1 proteins, in keeping with the observation that knockout of this gene in mouse embryos does not disrupt and, therefore, is not necessary for testis development (44). Rather, these mutations have gain-of-function effects, causing increased phosphorylation of the downstream targets, p38 and ERK1/2, and increased binding of cofactors RHOA, MAP3K4, FRAT1, and AXIN1. These downstream effects tilt the balance of gene expression in the testis-determining pathway causing decreased expression of *SOX9* and its downstream targets, FGF9 and FGFR2, and increased expression of β -catenin and its downstream target, FOXL2.

Unlike *Map3k1*, *Map3k4* is necessary for testis development as demonstrated by inheritance of homozygous truncation mutations in mouse embryos (45). The effect of these mutations is to diminish the transcription of *Sry*, an effect that is mediated by the *Map3k4* binding partner, *Gadd45 γ* , and by subsequent phosphorylation of p38 α , p38 β , and *Gata4* (46, 47). Homozygous loss of function mutations in *MAP3K4* have not been described in humans because these are likely to be early embryonic lethal. Nonetheless, overexpression of *MAP3K4* may have an additional role in testis determination, beyond that identified in knockout mice, mediating not only the initial expression of *SRY*, but also the subsequent expression of *SOX9*. This effect on *SOX9* expression was demonstrated in cotransfection experiments in which *MAP3K4* rescued the effects of *MAP3K1* mutations in human cells, normalizing the expression of *SOX9* and β -catenin (43).

These observations led to the development of a model for the role of the MAPK pathway in promoting sex determination (Figure 2). This model includes multiple genes (*SRY*, *RAC1*, *MAP3K4*, and *AXIN1*) that all promote testis determination through the up-regulation of *SOX9* and, through a feed-forward loop, FGF9. In turn, several of these genes (*SOX9*, *AXIN1*, and *GSK3 β*) create a block to ovarian development by destabilizing β -catenin. During ovarian determination, phosphorylated p38 and ERK1/2, and FOXL2 down-regulate the expression of *SOX9* and, thus, the resulting feed-forward loop and the block to ovarian development. Phosphorylated p38 and ERK1/2 and *AXIN1* (via destabilized *GSK3 β*) and *FRAT1*

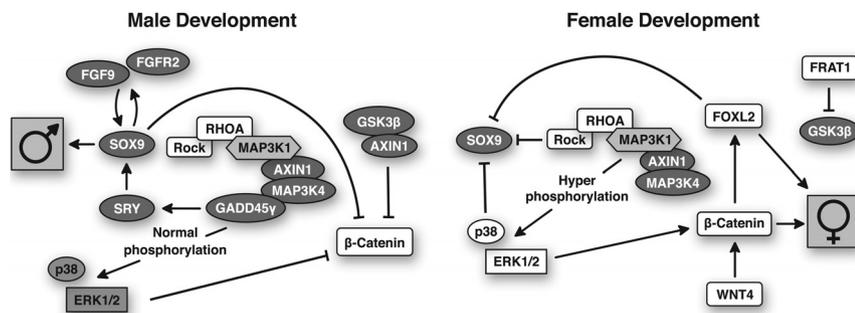


Figure 2. MAP3K1 cofactors and downstream targets play roles in promoting and blocking gonadal determination. Testis-promoting factors are shown in black, and ovary-promoting factors are shown in white. Factors that promote the action of a downstream target are shown as lines that end in arrows. Factors that block the action of a downstream target are shown as lines ending in bars. Male development: SRY, RAC1, MAP3K4, and AXIN1 all promote the expression of SOX9 and, through a feed-forward loop, FGF9. MAP3K4, through its cofactor, GADD45 γ , promotes the expression of SRY. SOX9, AXIN1, and GSK3 β promote the destabilization of β -catenin and, thus, create a block to ovarian development. Female development: RHOA, phosphorylated p38 and ERK1/2, and FOXL2 down-regulate the expression of SOX9 and, thus, the resulting feed-forward loop and block to ovarian development. Phosphorylated p38 and ERK1/2 and AXIN1 (via destabilized GSK3 β) and FRAT1 promote the stabilization of β -catenin and the up-regulation of the downstream targets, FOXL2 and FST. Possible sequestration of AXIN1 and MAP3K4 onto mutant MAP3K1 enhances β -catenin stabilization. Gain-of-function mutations in the MAP3K1 gene mimic the ovarian-determining pathway, overriding the testis-determining signal from an expressed, wild-type SRY gene.

also promote the stabilization of β -catenin and the up-regulation of its downstream targets, FOXL2 and FST. Gain-of-function mutations in the *MAP3K1* gene mimic the ovarian-determining pathway, overriding the testis-determining signal from an expressed, wild-type *SRY* gene.

Role of New Technologies in Identification of Gonadal DSDs

With the advent of fully mapped and subsequently fully sequenced genomes, new technologies have been developed that facilitate analyzing the whole genome or regions of the genome for copy number variants, structural rearrangements, single nucleotide variants, and short insertions and deletions (termed, “indels”). Array CGH uses oligonucleotide probes to detect submicroscopic duplications and deletions that are not visible by light microscopy (typically < 3 Mb). When applied to DSDs, array CGH may detect pathogenic variants or nonpathogenic variants that are found among unaffected normal individuals. Sorting between these possibilities requires replication among other affected individuals and identification of duplicated or deleted genes that represent plausible candidates for gonadal development.

Using array CGH, copy number alterations were identified for both known and novel genes. Among the known genes identified were deletion upstream of *SOX9* in a case of 46,XY DSD (48), deletions involving *NR5A1* in multiple cases of 46,XY DSD (49, 50), and partial deletion of *DMRT1* in a case of 46,XY ovotesticular DSD (51). Du-

plication of the X chromosome involving the *SOX3* and, in one instance, a larger chromosomal region have been found in three cases of 46,XX testicular DSD (22, 52). The cases support the hypothesis that overexpression of *SOX3* can substitute for expression of *SRY* to cause testicular development. Among the novel genes identified were duplication of a chromosomal region containing *PIP5K1B*, *PRKACG*, and *FAM189A2* in a case of 46,XY gonadal dysgenesis (53), duplication of the *SUPT3H*, and a deletion of *C2ORF80* in a pair of siblings with 46,XY gonadal dysgenesis (53), and a multiexon deletion of *WWOX* in a case of 46,XY DSD (54). A study of 23 patients with 46,XY gonadal dysgenesis identified likely causal copy number alterations in three patients,

yielding sensitivity of 13% (55).

The more recent and potentially greater impactful technological change has been the sequencing of all or part of individual genomes that can detect single nucleotide variants and indels (usually defined as < 1 kb) (56, 57). When performed at high density, sequencing can also detect copy number alteration. Two recent innovations—targeted capture or selective amplification of genes, and next-generation sequencing based on short-read, high-density coverage of the fragments—have made sequencing of individual genomes, exomes, or gene panels affordable and accessible for research and, potentially, clinical applications (58–60). The premise for sequencing approaches for monogenic disorders has been the fact that 85% of previously identified casual variants were identified in exons or at splice-junction boundaries in introns (61).

A custom capture and sequencing kit was developed to test the coding sequences of 35 genes known to be involved in sex determination (62). This kit was applied to a group of seven patients with a known genetic cause for their disorder who had received a genetic diagnosis and accurately identified the cause in every case. This group included a patient with Turner syndrome (45,X) and Klinefelter syndrome (47,XXY), indicating that chromosomal aneuploidies could be identified by sequencing. A genetic cause was found in two of an additional seven patients for whom a genetic cause had not been found previously. From their study, the authors proposed that genetic testing with array CGH and sequencing might serve as a first step in the evaluation of patients with DSDs,

before undertaking nonurgent hormonal, metabolic, and sonography tests (62). Indeed, they have abandoned their kit in favor of whole exome sequencing, while recognizing that false positives may be called in the process of analyzing sequencing data (63). Although these genetic approaches will gain traction in clinical practice, a clinical trial of “genes first” vs “hormones and sonogram first” should be undertaken to compare time to accurate diagnosis.

Whole exome sequencing has been applied to identify the genetic basis for Perrault syndrome, a condition of 46,XX ovarian dysgenesis, hearing loss, and, sometimes, mild intellectual disability and cerebellar and peripheral nervous system involvement. In one family of mixed European origin, two sisters with Perrault syndrome were found to be compound heterozygotes for mutations in *HSD17B4*, a gene that encodes 17 β -hydroxysteroid dehydrogenase type 4, a multifunctional peroxisomal enzyme involved in fatty acid β -oxidation and steroid metabolism. These compound heterozygous mutations (p.Y271C and p.Y568X) resulted in severely reduced expression of the HSD17B4 protein (64). Two other families with Perrault syndrome were found to have mutations in the *LARS2*, the gene that encodes mitochondrial leucyl-tRNA synthetase. A consanguineous Palestinian family had a homozygous p.Thr522Asn mutation, and a Slovenian family was compound heterozygous for c.1077delT frameshift and p.Thr629Met missense mutation (65). *HARS2*, the gene encoding histidyl tRNA synthetase has also been found to harbor mutations that cause Perrault syndrome, demonstrating the role for mitochondria for maintaining ovarian function and hearing (66).

Functional variant assays (FVAs) that couple flow cytometry to optically labeled antibodies were developed to address the need for high throughput, quantitative immunoassays that assess the phenotypic effects of genetic variants on protein quantification, post-translational modification, and interactions with other proteins, initially in the *MAP3K1* gene (42, 43). FVA currently comprises two methods. The digital cell Western uses permeabilized fixed cells followed by fluorescent probes annealing at room temperature and rapidly assessed with modified flow cytometry to quantify proteins and their post-translational modifications; each cell is measured independently for its protein expression level based on individual intensity. Then, collectively 100 000 to a million data points from each sample are normalized and statistically calculated as a digital value, hence digital cell Western. The protein coimmunoprecipitation assay binds a specific protein complex to an antibody-coupled bead matrix and then quantifies the interactions with that protein and its various partners all in one well. These FVA assays are rapid, quan-

titative, low-cost, and modular multianalyte assays. The aggregate large number of data points that were collected for each assay assured that the results were highly quantitative with narrow SD values. Analysis could be performed on multiple binding partners simultaneously, which was not formerly possible. Through these types of analyses, three types of *MAP3K1* variants were observed – *full gain-of-function mutations*, whose molecular effects exceeded a threshold and produced a physical phenotype; *partial gain-of-function alleles*, whose molecular effects exceeded a threshold for some targets, but not others, and did not produce a recognized physical phenotype; and *normal variants*, whose activities were constant for the various assays over the multiple controls.

Conclusion

Considerable progress has been made over the past 20+ years since genetic mechanisms involving *SRY* were shown to cause both 46,XX testicular DSD and 46,XY partial and complete gonadal dysgenesis. The repertoire of genes has been built out, and their organization into ovarian- and testicular-determining pathways that can block each other has been identified. Robust new technologies have been developed to evaluate the function of mutations in known and previously undescribed sex-determining genes, as well as to evaluate the phenotypic effects of these mutations. Because this repertoire of genes can account for only a fraction of the genetic causes, more genes are likely to be identified by sequencing and informatics. Moreover, epistatic interactions between variant-bearing genes will be identified that account for phenotypic variation in the presentation of these disorders.

Acknowledgments

The author thanks Johnny Loke, MS, for technical discussions and assistance with figure drawing.

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Disclosure Summary: The author has nothing to report.

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