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Review

Human sex-determination and disorders of sex-development (DSD)

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ABSTRACT

Several new genes and pathways have been identified in recent years associated with human errors of sex-determination or DSD. *SOX* family gene mutations, as well as mutations involving *GATA4*, *FOG2* and genes involved in MAP kinase signaling have been associated with virilization in 46,XX individuals or with 46,XY gonadal dysgenesis. Furthermore, mutations involving another key gene in sex-determination, *NR5A1*, are now known to be an important cause spermatogenic failure in the male and ovarian insufficiency in the female. These new findings offer insights into human sex-determination and highlight important differences between the human and mouse model.

This review will critically examine the evidence linking gene mutations, especially *MAP3K1*, to non-syndromic forms of human 46,XY gonadal dysgenesis or XX testicular/ovotesticular.

Contents

1. Introduction

DSD, defined as 'congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical' encompasses a wide spectrum of phenotypes [1]. This definition includes errors of primary sex-determination; 46,XY complete or partial gonadal dysgenesis (CGD, PGD; complete or partial absence of testis-determination) or 46,XX testicular DSD which refers to a male with testis and a normal male habitus and 46,XX ovotesticular DSD refers to individuals that have both ovarian and testicular tissue in the gonads. Our understanding of the genes involved in sex-determination and the mechanisms involved has improved dramatically over the past 10 years, however in cases of DSD a molecular diagnosis is still only made in only around 20% of DSD (excluding those cases where the biochemical profile indicates a specific steroidogenic block) [1]. Current data indicate that causal gene mutations can be found in around 50% of the patients who have errors of primary sex-determination. This review will focus on the gene mutations that result in human pathologies of primary sex-determination.

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2. 46,XY gonadal dysgenesis

2.1. SRY and SOX9

Approximately 15% of all cases of 46,XY CGD carry mutations in the Y-linked testis-determining gene *SRY* with the majority of these mutations localized within the HMG-domain [2]. A few rare cases of gonadal dysgenesis with small interstitial deletions 5′ and 3′ to the *SRY* open-reading frame have also been described [3,4]. In most cases the *SRY* mutations are *de novo* but some are inherited from an apparently normal and fertile father. Functional studies suggest that these inherited *SRY* mutations are hypomorphs that show partial biological activity compared to the baseline properties of wild-type protein [5]. Thus, in these familial forms the incomplete penetrance could be caused by stochastic effects around a threshold level of biological activity required for testis formation. In one exceptional case a *de novo* p.Gln2Ter mutation was reported in a woman with premature menopause [6]. The patient reported menarche and normal breast development at age of 13–14 years and had regular monthly menses until age 17, when she began oral contraceptives until she was 25 years. At that time she developed irregular menses that continued for 2 subsequent years as she attempted to get pregnant. This suggests that when the human gonad cannot form a testis it tries to develop as an ovary. Usually, in XY gonadal dysgenesis, the ovarian tissue degenerates during early development or post-natally to form a streak of fibrous tissue, but in some individuals the ovarian tissue persists until puberty or beyond.

Campomelic dysplasia (CD), characterized by skeletal defects and typical facial appearance, is associated testicular dysgenesis in about 75% of affected XY individuals [7]. *Sox9* plays both an essential role in the specification and differentiation of mesenchymal cells toward the chondrogenic lineage through transcriptional modulation of *Col2a1*, the major matrix protein of the mature cartilage as well as establishing Sertoli cell identity in the developing testis immediately following the expression of SRY. Many mutations have been reported in *SOX9* associated with CD and more recent studies have focused on potential regulatory elements that may also cause DSD. The developmental timing and tissue-specific transcriptional regulation of *SOX9* is highly complex and involves multiple elements located in flanking regions of at least ∼1 Mb upstream and 1.6 Mb downstream. In the upstream region, translocations and inversion breakpoints associated with CD fall within two clusters located ∼400 kb apart [8]. Patients with these rearrangements generally have a milder phenotype than the intragenic mutations [8,9]. Large (>1 Mb) duplications 5′ to *SOX9* that may lead to *SOX9* misexpression are associated with brachydactyly-anonychia (symmetric brachydactyly of the hands/feet, hyponychia or anonychia) [10]. Pierre Robin sequence, a craniofacial disorder characterized by micrognathia, cleft palate and macroglossia with normal testis development in 46,XY cases is associated with a 75 kb deletion located 1.38 Mb upstream and a deletion located 1.56 Mb downstream of *SOX9* [11].

A testis-specific enhancer *Sox9* has been mapped in mice to a 1.4 kb core region termed *Tesco* that is located 13 kb upstream from *Sox9* [12]. Both Sry and Nr5a1 (see Section 2.2) bind to the *Tesco* enhancer sequence *in vivo*, possibly through a direct physical interaction to up-regulate *Sox9* gene expression. Once Sox9 protein levels reach a critical threshold, several positive regulatory loops are initiated for its maintenance, including auto-regulation of its own expression and formation of feed-forward loops *via* Ffg9 or Pgd2 signaling [12]. Other cofactors are likely to be involved in this process but have not yet been identified. The homologous human SRY-responsive enhancer can also be activated by human SRY and SOX9 together with NR5A1 suggesting that there may be a conserved mechanism for male-specific up-regulation and maintenance of SOX9 expression in gonadal pre-Sertoli cells in human and mouse. To date, mutations involving the *TESCO* element have not been reported in association with human DSD.

Rearrangements grouped around a 600 kb locus (termed *RevSex*) upstream of the human *SOX9* gene are associated with both XY and XX DSD. Five cases of 46,XX testicular or ovotesticular DSD that carried duplications of this region and a familial case of 46,XY DSD that carried a deletion have been reported [13–15]. We identified three phenotypically normal patients presenting with azoospermia and 46,XX testicular DSD [16]. This included two brothers, who carried a 83.8 kb duplication that refined the minimal region associated with 46,XX-*SRY* negative DSD to a 40.7–41.9 kb element, which contains two predicted enhancer motifs. A proximal strong enhancer motif, which is enriched for H3K4 methylation and H3K27 acetylation, both of which are epigenetic marks that are characteristic of gene activation. The histone acetyltransferase EP300, which regulates transcription *via* chromatin remodeling binds to this element. In mice, *Ep300* is strongly expressed in the somatic cell lineages of both the XX and XY gonad during sex-determination and it can act as a co-activator of both Nr5a1 and Sox9 [17]. This enhancer motif is located between two predicted binding sites for DMRT1-binding (see Section 2.7). There is also data suggesting that deletions of an immediately adjacent and non-overlapping region are associated with 46,XY gonadal dysgenesis [18]. In our experience about 10% of cases of testicular/ovotesticular DSD and 46,XY gonadal dysgenesis have rearrangements involving the *RevSex* locus.

2.2. NR5A1

A major cause of human DSD is mutations involving the *NR5A1* gene. NR5A1 belongs to the subfamily of transcription factors known as nuclear receptor subfamily 5 (group A, member 1; NR5A1), which is highly conserved in vertebrates [19]. Like other nuclear receptors, the NR5A1 protein consists of a DNA-binding motif composed of two zinc-chelating modules that coordinate the interaction between the receptor and hormone response element [20]. NR5A1 binds DNA as a monomer, with DNA-binding stabilized *via* a 30 amino acid extension to the DNA-binding domain (Ftz-F1 or A box). The C-terminal ligand-binding domain (LBD) encompasses an AF-2 domain that cooperates with a proximal activation domain (AF-1) and is required for maximal biological activity with co-activators such as NCOA1 (SRC-1) [20]. Posttranslational modification plays an important role in modulating NR5A1 activation and repressor functions. Phosphorylation of Ser203 within the LBD enhances the interaction of the cofactors Tif2 (Grip1/Ncoa2) and Smrt (Ncor2/Trac1) with the AF-1 and AF-2 motifs. Strong transcriptional repression requires sumoylation of lysines Lys119 and Lys194, which increases interactions with DEAD box proteins including Ddx20 [21].

XY mice lacking *Nr5a1* have gonadal dysgenesis resulting in male-to-female sex reversal significantly diminished corticosterone levels, elevated adrenocorticotrophic hormone levels due to adrenal insufficiency and underdevelopment of the spleen and impaired clearance of abnormal red blood cells [22]. *Nr5a1*$^{-/-}$ mice do not express luteinizing hormone (LH) or follicle stimulating hormone (FSH) from the gonadotroph cells in the pituitaries, whilst the VMH is disorganized [23]. Mice lacking *Nr5a1* specifically in the VMH show high fat diet-induced obesity and increased anxiety-like behavioral patterns [24]. In the human, mutations involving *NR5A1* are associated with a wide range of reproductive anomalies including adrenal insufficiency and 46,XY gonadal dysgenesis, ambiguous genitalia, hypospadias, micropenis, spermatogenic failure with normal genitalia and primary ovarian insufficiency [25].

In a study of 315 men with spermatogenic failure, we identified heterozygous missense mutations in *NR5A1* in seven men with either azoospermia or severe oligozoospermia [26]. Testis

histology in one man with azoospermia was suggestive of a mild form of testicular dysgenesis rather than Sertoli Cell Only Syndrome. In all cases the men carrying the *NR5A1* mutations had normal development of the external genitalia [26]. Other studies have confirmed these findings and current data indicate that around 3% of men with spermatogenic failure carry mutations in the gene (*e.g.* [27] and unpublished data). The phenotype variability associated with these mutations has not been explained. In some cases it may be a consequence of the specific amino acid involved. For example, a homozygous p.R103Q NR5A1 mutation was reported in a child with severe 46,XY DSD and absent spleen. The mutation decreased the ability of the mutated NR5A1 protein to transactivate *TLX1*, a transcription factor that is essential for spleen development [28]. The R103Q mutation impaired activation of steroidogenic genes, without affecting synergistic NR5A1/SRY co-activation of *SOX9*. In this case the functional analysis are consistent with the phenotype of the patient. However, phenotypic variability in other cases is more difficult to explain. For example, the linked variants p.Gly123Ala + p.Pro129Leu are found mainly in North African populations and they are associated with either male infertility, female infertility or 46,XY DSD. It is likely that some of these patients carry novel or rare variants in other genes involved in sexual development. Our exome sequencing studies suggest this may be the case since we have identified patients with DSD, who carry *NR5A1* mutations together with novel or rare variants in for example *MAP3K1* or known cofactors of NR5A1 such as *NRIP1* (*RIP140*, unpublished data).

2.3. The cofactors GATA4 and FOG2

GATA4, cooperatively interacts with several proteins including NR5A1, to regulate the expression of genes critical for testis-determination and differentiation [29]. *Gata4*^{ki} mice, which carry a p.Val217Gly mutation in the N-terminal zinc finger domain of the protein that abrogates the physical interaction of Gata4 with the cofactor Fog2, present with severe testicular dysgenesis [30–32]. A proportion of XY patients carrying deletions of human 8p23.1 that includes the *GATA4* gene have genitourinary anomalies such as hypospadias and bilateral cryptorchidism ([33] and references therein). We identified a *GATA4* (p.Gly221Arg) heterozygous mutation that was associated with DSD in 46,XY individuals and congenital heart disease that affected both 46,XX and 46,XY individuals [34]. The p.Gly221Arg residue is immediately adjacent to the mouse p.Val217Gly *Gata4*^{ki} mutation in the N-terminal zinc finger [31,34]. The p.Gly221Arg variant failed to bind to and to transactivate the *AMH* promoter. It also disrupted the synergistic activation of the *AMH* promoter by GATA4 and NR5A1 proteins. The GATA4p.Gly221Arg protein lacked the ability to bind to the FOG2 protein [34]. Recently, we have identified other heterozygous missense mutations in *GATA4* including in individuals with no evidence for heart disease suggesting that mutations involving the gene may be a more frequent cause of XY DSD that currently realized (unpublished data). Since mutations in *GATA4* are associated with errors of testis-determination, we examined the possibility that the protein partner of GATA4, FOG2 may also be involved. FOG2 (also known as ZFPM2) is a zinc finger cofactor that binds to the N-terminal zinc fingers, thereby modulating the transcriptional activity of GATA factors depending on the cellular and promoter context. A role for Fog2 in sex-determination is shown by XY *Fog2*$^{-/-}$ mice, which fail to develop testis and where the expression of key genes involved in testis formation (*Sry*, *Sox9*, and *Amh*) is reduced. A role for FOG2 in human testis determination was suggested by a boy with congenital heart disease and 46,XY gonadal dysgenesis, who carried a balanced t(8;10)(q23.1;q21.1) translocation with a breakpoint that mapped within the *FOG2* gene [35]. Furthermore, Tan et al. [36] described an individual with multiple congenital anomalies and gonadal dysgenesis in association with a *de novo* chromosomal translocation 46,XY t(8;18)(q22;q21). Using exome sequencing we identified two patients, both with 46,XY gonadal dysgenesis, who carried missense mutations in the *FOG2* gene [37]. In both the families, there was no patient or family history of cardiac anomalies. In one family a FOG2 p.S402R mutation was identified. The mutated protein, which lacked the ability to bind to the GATA4 protein, was carried by the unaffected mother and maternal grandmother. A second patient carried an inherited homozygous p.M544I mutation and a *de novo* heterozygous p.R260Q mutation. The p.M544I variant by itself had little effect on the biological activity of FOG2 protein in transactivation of gonadal promoters, but a combination of both the p.R260Q and the p.M544I variants eliminated its interaction with the GATA4 protein [37].

2.4. Hedgehog signaling and DSD

Desert Hedgehog (*DHH*), one of the three mammalian *Hedgehog* (*Hh*) genes, is expressed in the developing testes and Schwann cells of peripheral nerves and it is involved in establishing murine fetal Leydig cells in the mouse [38]. Hh signaling may operate *via* Nr5a1 to transform precursor cells into the fetal Leydig cell lineage since the ectopic activation of Hh signaling in Nr5a1-positive somatic cell population of the mouse ovary results in the development of Leydig-like cells with virilized external genitalia [38]. Human 46,XY PGD together with extensive formation of minifascicles within the endoneurium of the sural nerve was observed in association with a homozygous missense mutation at the initiating codon of *DHH* [39]. Although four further homozygous mutations in *DHH* have been reported in association with DSD with no apparent signs of polyneuropathy. However, the polyneuropathy can be difficult to detect and may have been overlooked in these patients. A recent study described a novel homozygous R124Q mutation associated with 46,XY gonadal dysgenesis, seminoma formation and neuropathy with minifascicle formation [40]. The role of the DHH signaling is to establish the fetal Leydig cell lineage and, as suggested by Werner et al., the gonadal dysgenesis that is observed in the patients carrying homozygous mutations may be due to impairment of Sertoli cell–Leydig cell interaction during early testis formation [40].

The hedgehog acyl-transferase (HHAT) protein, a member of the MBOAT family of membrane-bound acyltransferases, catalyzes amino-terminal palmitoylation of Hh proteins. Palmitoylation is crucial for biological activity and plays a major role in guiding Hh proteins to specific membrane domains. Recently, a homozygous G287V missense mutation in the MBOAT domain of HHAT was reported in a single sporadic case of 46,XY gonadal dysgenesis and chondrodysplasia [41]. The mutation disrupted the ability of HHAT protein to palmitoylate Hh proteins including DHH and SHH suggesting that it is pathogenic. Hhat is expressed in the somatic cells of both XX and XY gonads at the time of sex determination [41]. In mice the absence of *Hhat* in the XY gonad did not affect testis-determination but from E12.5 to E15.5 there was a reduction in testis size. Sertoli cell specification appeared to be normal but the development of fetal Leydig cells, as well as the proper formation of testis cords, was severely impaired, leading ultimately to testicular dysgenesis. These data suggest that HHAT is not involved in primary testis-determination but that it is required for the initiation of Leydig cell formation. It is unlikely that mutations in *HHAT* are a common cause of DSD. The phenotype of the girl carrying homozygous mutation is a complex, consisting of a rare combination of gonadal dysgenesis and chondrodysplasia [41]. Moreover, a *de novo* dominant mutation the MBOAT domain of *HHAT* was reported in association with intellectual disability and apparently normal testis

development [42] suggesting that the *HHAT* genotype–phenotype relationship is likely to be complex.

2.5. CBX2

Mice lacking *Cbx2* display posterior transformation of the vertebral columns and sternal ribs, failure of T-cell expansion and XY mice show male-to-female sex reversal whereas XX animals have either absent or smaller ovaries [43]. A single mutation in the human *CBX2* gene has been reported. This was a 46,XY girl who carried two independent mutations in *CBX2* – a paternally inherited p.Pro98Leu mutation and a maternally inherited p.Arg443Pro mutation [44]. Histology of the gonads at 4.5 years revealed apparently normal ovaries. Although polycomb group proteins are traditionally regarded as transcriptional repressors, there is evidence that at least in some cellular or promoter contexts CBX2 acts as a transcriptional activator of *NR5A1* and *SRY* [43,44]. In the patient described above presence of apparently normal ovaries suggests that CBX2 actively represses fetal ovarian development in an XY individual.

2.6. Map kinase signaling and 46,XY DSD

The mitogen-activated protein kinases (MAPKs) are activated through an evolutionarily conserved three-component signal transduction cascade, composed of a mitogen-activated protein kinase kinase kinase 1 (MAP3K1), a MAP2K and a MAPK. A role for the MAPK signaling pathway in mammalian sex-determination was indicated by the identification of a mutation in murine *Map3k4* in a forward genetic screen [45]. XY embryos lacking functional *Map3k4* on a predominantly C57BL/6J background exhibit embryonic gonadal XY sex-reversal [45]. Mice lacking *Gadd45g*, which encodes a protein that interacts with Map3k4, also show a lack of testis-determination [46]. Sex-reversal is associated in the former with a either failure to transcriptionally up-regulate *Sry* and in the latter with a delay in *Sry* expression. Furthermore, work by Greenfield and colleagues demonstrated that the absence of both the p38a and p38b MAPK isoforms results in XY sex-reversal that is also associated with reduction in *Sry* levels [47]. Therefore, at least in mice, available data are consistent with a GADD45γ/MAP3K4/p38 pathway is required for initiating the appropriate expression of *Sry* in the XY gonad at sex-determination. In the human, the role of MAP kinase signaling in testis-determination has not been resolved. Mutations in a different MAP kinase from that seen in mouse, *MAP3K1* have been identified in cases of 46,XY DSD [48]. Six candidate pathogenic mutations have been described (p.Gly616Arg, p.Leu189Pro, p.Leu189Arg, c.634-8T>A, p.Pro153Leu and c.2180-2A>G) together with functional analysis. The MAPK signaling activity in transformed B-cell lymphoblastoid cell lines from the patients cell lines enhanced phosphorylation of the MAPKs p38 and ERK. RHOA, a known positive regulator of MAP3K1 kinase activity, exhibited increased binding to protein complexes containing mutant MAP3K1s. The authors conclude that these mutant versions of MAP3K1 proteins appear to behave like gain-of-function alleles, enhancing functionality of the encoded protein [48]. However, the relationship between the postulated pathogenic *MAP3K1* mutations and the phenotype has not been established. The Gly616Arg mutation, seen in one family, is predicted not to interfere with protein function by PolyPhen and SIFT. This variant has been reported in the database dbSNP138 at a frequency of 1:834 alleles in European Americans. Similarly, the Pro153Leu mutation is not predicted to be damaging to the protein by either PolyPhen or SIFT and it is present at a frequency of 1:162 alleles in South Asian populations (1 in 56 alleles of Bengali populations) (dbSNP138). Given these frequencies, it is highly unlikely that either of these mutations are, by themselves, pathogenic. Furthermore, the Leu189Pro mutation that was reported may have been inherited from the father. These observations also question the utility of the MAP3K1 assay that uses transformed lymphoblastoid cell lines and whose relevance to Sertoli cell development is questionable. The Pro153Leu variant, for example, of all of the mutants studied exhibits some of the strongest alterations the binding of RHOA, MAP3K4, FRAT1, and AXIN1 and phosphorylation of ERK1/2 in transformed B-cell lymphoblastoid cell lines. These assays by themselves are therefore unlikely to distinguish between inconsequential rare (or common) variants and pathogenic mutations.

These patients, including the family we identified and that was used to map the *MAP3K1* mutation [49] show no other apparent phenotypic anomalies other than 46,XY gonadal dysgenesis (unpublished data).

Interestingly, mice lacking *Map3K1* do not show male-to-female sex-reversal. These mice are viable and fertile but show somewhat reduced numbers of Leydig cells and an increased embryonic gonadal length [50,51]. This suggests that either the MAP kinase signaling pathways in human or mouse have diverged or that the difference in phenotype is caused by an intrinsic difference in the type of mutation. Mice generated by gene targeting have loss-of-function *Map3k1* alleles whereas the *MAP3K1* mutations that are associated with 46,XY DSD are either splice site mutations, missense mutations or in-frame deletions that possibly act as gain-of-function alleles ([48], and unpublished data). To date the role of MAP3K1 in human sex-determination is unknown and downstream effectors of MAP3K1 in the human developing testis have not been identified. The phenotype of 46,XY complete gonadal dysgenesis is similar to that associated with mutations in *SRY* itself suggesting that *MAP3K1* mutations are impacting the early stages of testis-determination.

2.7. DMRT1, an evolutionary conserved sex-determining gene

Deletions of the terminal 9p are associated with monosomy 9p syndrome, which is characterized by mental retardation together with a characteristic series somatic anomalies [52]. Similar to individuals carrying *SOX9* mutations ~70% of 46,XY patients show various degrees of gonadal dysgenesis. Two DMRT genes, *DMRT1* and *DMRTA3* genes, which are orthologues of the doublesex (dsx) of Drosophila and mab-3 of Caenorhabditis, are located within the minimal recurrently deleted region [53]. *Dsx* controls the terminal switch of the pathway leading to sex fate choice in Drosophila and *mab-3* is necessary to confer male traits in *C. elegans*.

The role of DMRT1 in mammalian sex-determination is unclear. Although *Dmrt1* is not required in the mouse for testis determination, its continuous expression in the adult testis is required to maintain organ identity, because forced attenuation of *Dmrt1* expression in adult testis results in the testis changing to an ovary [54]. Although deletions of 9p24 suggest that *DMRT1* hemizygosity is sufficient in some individuals to lead to a failure of testicular development, these deletions usually remove other genes, including the evolutionary-related *DMRT2* and *DMRT3* genes. The DMRT1 protein binds DNA *in vivo* as a tetramer, trimer or dimer [55]. This discovery is particularly important because specific mutations in the DM-domain that cause an intersex phenotype in Drosophila show a reduction or absence of DNA-binding. Using an exome sequencing approach, we identified a *de novo* missense mutation in the DM DNA-binding domain of *DMRT1* associated with 46,XY complete gonadal dysgenesis [55]. There were no other somatic anomalies in this girl. The histology of the gonad was similar to that of an *SRY* mutation and showed no evidence of testicular material suggesting that the mutation was affecting primary testis-determination. *In vitro* studies indicated that the mutant protein had reduced DNA affinity, altered sequence specificity and when mixed with wild-type protein, it bound as a tetramer

complex to an *in vitro* Sox9 DMRT1-binding site that is bound as a trimer by the wild-type protein [55]. This suggests that the lack of testis-determination seen in this patient is due to a combination of haploinsufficiency and a dominant disruption of normal binding stoichiometry at biologically relevant target binding sites. This combination of dominant negative activity and haploinsufficiency may be specific for this amino acid change and may explain the lack of *DMRT1* mutations associated with human sex-reversal reported to date in the literature.

3. SOX genes and 46,XX testicular and ovotesticular DSD

SRY and the X-linked gene *SOX3* are thought to share a common ancestor and during early mammalian evolution *SRY* arose from a gain-of-function mutation in the proto-Y allele of *SOX3* that resulted in testis specific expression. Although *Sox3* is normally not expressed in the urogenital ridge at the moment of sex-determination, it can substitute for *Sry* in testis determination. This is demonstrated by the ectopic expression of *Sox3* in XX hemizygous transgenic gonads that induces *Sox9* upregulation and consequent testicular development [56].

In the human *SOX3* loss-of-function mutations are not associated with either 46,XY gonadal dysgenesis nor 46,XX testicular/ovotesticular DSD, but are associated with mental retardation and growth hormone deficiency [57]. However, chromosomal rearrangements at the *SOX3* locus can lead to testis development in an XX background. A screen of 16 46,XX *SRY*-negative testicular DSD patients, one patient carried two microduplications of approximately 123 kb and 85 kb, the former of which spanned the entire *SOX3* gene whereas the other was located 350 kb proximal to *SOX3*, a second patient carried a single 343 kb microdeletion immediately upstream of *SOX3* and a third XX male with multiple congenital anomalies carried a 6-Mb duplication including *SOX3* and at least 18 other genes [56]. These data strongly suggest that ectopic gonadal expression of *SOX3* is responsible for the phenotype rather than gene dosage. In this model, the inappropriate expression of SOX3 protein can replace the expression of SRY/SOX9 proteins in an XX individual. SOX3 can transactivate a human TES-like enhancer sequence upstream of *SOX9* suggesting that this may be the mechanism [56]. Haines et al. described an XX male who carried 774-kb insertion translocation from chromosome 1 into a palindromic sequence 82 kb distal to *SOX3* [57]. Two further duplication of the *SOX3* gene have been reported in XX males [58,59]. These studies raise the possibility that ectopic expression of other HMG-box containing proteins in the urogenital ridge at the moment of sex-determination may result in testicular development in a chromosomal female individual. One such example may be *SOX10*. Complete or partial duplications of chromosome 22 in 46,XX-*SRY* negative individuals are associated with various degrees of masculinization [60–62]. Nicholl et al. described a 46,XX trisomy 22 male with micropenis, but the internal genitalia consisted of a small vagina and uterus with both testis-like gonads located in the abdominal cavity. An individual with ambiguous genitalia with one gonad consisting of ovarian tissue and the other showing testis-like structures was associated with a 46,XX,rec(22)dup(22q)inv(22)(p13q13.1)mat chromosome complement [62]. Further delimitation of the minimal region was demonstrated by a *de novo* duplication of 22q11.2–22q13 in a 46,XX *SRY*-negative male with mild hypospadias, dysmorphic features and hypotonia [63]. Overexpression of *SOX10* in the urogenital ridge could exceed a critical threshold level required for the initiation of testis development. This hypothesis is supported by the observation that transgenic expression of *Sox10* in the gonads of XX mice resulted in testis formation [64]. The overexpression of *SOX10* may compensate for the lack of *SOX9* expression in XX gonads since the SOX10 protein has the capacity to activate transcriptional targets of SOX9 [64]. Furthermore, in mice the absence of both *Sox9* (pro-testis) and *Rspo1* (pro-ovary) in XX fetuses results in the development of ovotestes and hypoplastic testis [65]. SoxE group genes, *Sox10* and *Sox8* that are normally repressed by Rspo1, are activated in the double knockout gonads and presumably compensate for the absence of *Sox9* [65]. Furthermore, when XX and XY $Rspo1^{KO} + Sox9^{KO}$ gonads were compared at the same stage, the XY gonads always appeared more masculinized opening the intriguing possibility that Sry may be inducing the expression of male-specific genes other than *Sox9*.

The short arm of the human X chromosome may harbor a gene involved in testis-determination since linkage analyses of 9 familial cases of 46,XY CGD that defined a 3.41 cM (75.79–79.2 cM) critical region proximal to *NR0B1* (*DAX1*) at Xp11.21–11.23 [66]. A large deletion of Xp has also been described in a case of 46,XX *SRY*-negative ovotesticular DSD [67]. NR0B1 can both as a co-repressor and co-activator with NR5A1 depending on the cellular and promoter contexts. *NR0B1* loss-of-function mutations are associated with adrenal hypoplasia congenita and hypogonadotropic hypogonadism, whereas duplications of Xp21.3 or deletions that may dysregulate the expression of *NR0B1* are associated with 46,XY gonadal dysgenesis with or without multiple congenital anomalies ([68] and references therein).

4. *RSPO1, WNT4, FOXL2* and 46,XX *SRY*-negative testicular and ovotesticular DSD

Mutations involving *R-spondin1 (RSPO1)* and *WNT4* are associated with exceptionally rare syndromic forms of 46,XX testicular/ovotesticular DSD. RSPO1 belongs to a family of secreted furin-like domain containing proteins, which have pleiotropic functions in development and stem cell growth through their role in Wnt/β-catenin signaling. $Rspo1^{-/-}$ XX mice show partial female-to-male sex reversal possibly by antagonizing dickkopf-1 (Dkk1)-dependent internalization of LRP6, thereby resulting in a stabilization of cytoplasmic β-catenin [69,70]. Human homozygous *RSPO1* mutations are associated with a rare recessive syndrome, which is characterized by XX testicular DSD, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin [71]. Mutations involving *RSPO1* have not been reported in non-syndromic cases of testicular and ovotesticular DSD (unpublished data).

The absence of *Wnt4* in XX mice results in a partial masculinization of the gonad including the differentiation of some Leydig-like cells [72]. In the human, four dominant heterozygous missense mutations in *WNT4* have been reported in 46,XX women with various degrees of virilization including androgen excess and abnormal development of Mullerian ducts [73–76]. A single homozygous *WNT4* mutation was reported in a consanguineous family with an embryonic lethal syndrome of 46,XX testicular DSD and dysgenesis of kidneys, adrenals, and lungs (SERKAL syndrome; SEx Reversion, Kidneys, Adrenal and Lung dysgenesis) [77].

Heterozygous mutations of the human forkhead transcription factor *FOXL2* are associated blepharophimosis syndrome (BPES) a developmental disorder of the eyelids and the ovary [78]. The majority of mutations reported in *FOXL2* are dominant and heterozygous. Goats with polled intersex syndrome (PIS) carry a chromosomal deletion ~200 kb upstream of *FOXL2* that influences its transcription and homozygous carriers for the mutation show XX female-to-male sex reversal [79]. $Foxl2^{-/-}$ mice have ovaries that contain cords enclosing Sertoli-like cells and express male markers such as *Sox9* [80]. Targeted ablation of *Foxl2* in the adult ovary leads to transdifferentiation of the supporting cells of the ovary into the supporting cells of the testes that show

expression of SOX9 [81]. Two recessive mutations have been reported in the human associated with BPES and ovarian failure – a phenotype identical to the dominant heterozygous mutations with no evidence of virilization [82,83]. However in both cases it is likely that the mutant alleles are hypomorphic, retaining some biological activity so the phenotype of a true *FOXL2* null mutation in the human remains to be determined. The analyses of non-syndromic human 46,XX testicular or ovotesticular DSD has not identified mutations involving *FOXL2* [unpublished observations].

5. Conclusions

The last 5 years have seen considerable advances in our knowledge of the genetic causes of 46,XY and 46,XX DSD due to rapid developments in -omic technologies and a greater awareness of DSD as a public health issue. The latter is leading to improved details descriptions of the DSD phenotypes/families and more knowledge-based diagnosis. Technologies for genomic analyses, such as high resolution comparative genome hybridization or genome sequencing are both improving in accuracy and become more affordable. Consequently, this is translating into a surge of new genetic data pertaining to DSD.

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