NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development

Dorien Baetens, MSc1, Hans Stoop, PhD2, Frank Peelman, PhD3, Anne-Laure Todeschini, PhD4, Toon Rosseel, PhD1, Frauke Coppieters, PhD1, Reiner A. Veitia, PhD5, Leendert H.J. Looijenga, PhD2, Elfride De Baere, MD, PhD1 and Martine Cools, MD, PhD5

INTRODUCTION

Although several genetic mechanisms leading to 46,XY disorders of sex development (DSD) have been elucidated in recent years, little is known about the developmental pathways that may induce testicular development in 46,XX individuals when dysregulated. Approximately 80% of all 46,XX testicular DSD cases are explained by a translocation of the Sex Determining Region Y (SRY) gene.1,2 Duplications of SRY-box 9 (SOX9) or its regulatory region are a rare cause of 46,XX testicular DSD.3,4 Copy number variations disrupting the regulatory region of SRY-box 3 (SOX3), a putative ancestor of SRY, have also been found in patients with 46,XX DSD.3,5 In line with this, 46,XX DSD phenotypes have been associated with partial duplications of human chromosome 22q13 containing SRY-box 10 (SOX10).7,8 Studies in mice overexpressing Sox10 strongly implicate gain-of-function of SOX10 in 46,XX DSD.9 In addition to these gain-of-function changes of male sex-determining genes, loss-of-function mutations of female sex-determining genes can give rise to 46,XX DSD. This is illustrated by biallelic loss-of-function mutations in MAML1, a direct NR5A1 target previously associated with 46,XY DSD. In gonads of affected individuals, ovarian FOXL2 and testicular SRY-independent SOX9 expression observed.

Conclusions: We propose NR5A1, previously associated with 46,XY DSD and 46,XX primary ovarian insufficiency, as a novel gene for 46,XX (ovo)testicular DSD. We hypothesize that p.(Arg92Trp) results in decreased inhibition of the male developmental pathway through downregulation of female antitestis genes, thereby tipping the balance toward testicular differentiation in 46,XX individuals. In conclusion, our study supports a role for NR5A1 in testis differentiation in the XX gonad.

Keywords: gonadal development; NR5A1; ovotesticular DSD; testicular DSD; 46,XX DSD

NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development

Dorien Baetens, MSc1, Hans Stoop, PhD2, Frank Peelman, PhD3, Anne-Laure Todeschini, PhD4, Toon Rosseel, PhD1, Frauke Coppieters, PhD1, Reiner A. Veitia, PhD5, Leendert H.J. Looijenga, PhD2, Elfride De Baere, MD, PhD1 and Martine Cools, MD, PhD5

INTRODUCTION

Although several genetic mechanisms leading to 46,XY disorders of sex development (DSD) have been elucidated in recent years, little is known about the developmental pathways that may induce testicular development in 46,XX individuals when dysregulated. Approximately 80% of all 46,XX testicular DSD cases are explained by a translocation of the Sex Determining Region Y (SRY) gene.1,2 Duplications of SRY-box 9 (SOX9) or its regulatory region are a rare cause of 46,XX testicular DSD.3,4 Copy number variations disrupting the regulatory region of SRY-box 3 (SOX3), a putative ancestor of SRY, have also been found in patients with 46,XX DSD.3,5 In line with this, 46,XX DSD phenotypes have been associated with partial duplications of human chromosome 22q13 containing SRY-box 10 (SOX10).7,8 Studies in mice overexpressing Sox10 strongly implicate gain-of-function of SOX10 in 46,XX DSD.9 In addition to these gain-of-function changes of male sex-determining genes, loss-of-function mutations of female sex-determining genes can give rise to 46,XX DSD. This is illustrated by biallelic loss-of-function mutations in MAML1, a direct NR5A1 target previously associated with 46,XY DSD. In gonads of affected individuals, ovarian FOXL2 and testicular SRY-independent SOX9 expression observed.

Conclusions: We propose NR5A1, previously associated with 46,XY DSD and 46,XX primary ovarian insufficiency, as a novel gene for 46,XX (ovo)testicular DSD. We hypothesize that p.(Arg92Trp) results in decreased inhibition of the male developmental pathway through downregulation of female antitestis genes, thereby tipping the balance toward testicular differentiation in 46,XX individuals. In conclusion, our study supports a role for NR5A1 in testis differentiation in the XX gonad.

Keywords: gonadal development; NR5A1; ovotesticular DSD; testicular DSD; 46,XX DSD
Materials and Methods

Subjects
Case 1, the second child of two healthy, nonconsanguineous parents, was born after an uneventful pregnancy. Mild hypertrophy of the clitoris was noticed at birth. Vaginoscopy and laparoscopy detected the presence of a cervix and possibly a right hemiuterus, a left epididymis, and male vasculature. Both inguinal gonads were biopsied. The left gonad revealed testicular differentiation with germ cells present; the right gonad contained only fibrotic tissue. A human chorionic gonadotropin (hCG) stimulation test (1,500 IU administrated intramuscularly after blood sampling after 72 h) was performed on the seventh day after birth, resulting in a testosterone level of 2.52 nmol/l (baseline: 0.72 nmol/l); anti-Müllerian hormone (AMH) was between the male and female references (99.9 pmol/l). Based on these results and the child’s phenotype, a female sex assignment was made. Microarray-based comparative genomic hybridization (array CGH) showed a normal female pattern, and fluorescence in situ hybridization (FISH) with SRY-specific probes excluded SRY translocation. The results of quantitative polymerase chain reaction for the regulatory RevSex locus, upstream of SOX9, and MLPA for different sex development–related genes (P185 Intersex, MRC Holland, Amsterdam, The Netherlands) were normal.

Case 2 is a 22-year-old woman who had been diagnosed with 46,XX ovotesticular DSD. She had ambiguous genitalia at birth and a short, blind-ending vagina. At age 4, she underwent clitoroplasty and bilateral gonadectomy of abdominal gonads, which both turned out to be ovotestes, including primordial and primary follicles in the ovarian part and Sertoli cell–only tubules in the testicular part. SRY–specific FISH was negative, and array CGH revealed a small duplication reported to be a benign variant (hg19: chr 8: 132161619 – 132812388). RevSex locus-specific quantitative polymerase chain reaction was normal. She has one younger sister, who displayed normal puberty; family history was unremarkable.

Case 3 is a 23-year-old man with 46,XX testicular DSD. At birth, a micropenis, penile hypospadia, and bilateral scrotal but atrophic testes were noticed. Gonadal biopsies at the age of 2 years revealed bilateral testicular differentiation with immature seminiferous tubules without germ cells and limited intertubular fibrosis. When he was 12 years old, a prolonged hCG test (2 × 1,500 IU per week for 3 consecutive weeks) led to a peak increase of serum testosterone to 6.0 nmol/l. Hypergonadotrophic hypogonadism developed rapidly thereafter, and testosterone supplementation was started at the age of 13. FISH with SRY and TSPY probes was negative, and array CGH and quantitative polymerase chain reaction for the RevSex locus were normal. He is the oldest of three brothers; family history is unremarkable.

All three patients are Caucasian. Cases 1 and 3 are from East Flanders in Belgium, and case 2 originates from the south of the Netherlands, close to the Belgian–Dutch border. All families live near (within 70 km) each other. Pedigrees of the three families are shown in Figure 1a and phenotypic data for cases 1–3 are summarized in Table 1; phenotypic data for cases 4–9 are presented in the Supplementary Data online (Supplementary Table S1 online).

Genetic Study
Whole-exome sequencing (WES) was performed in nine patients with 46,XX (ovo)testicular DSD; in four additional patients, included later, the coding region of NR5A1 was sequenced. Enrichment for WES was performed with the SureSelectXT Human All Exon V5 kit (Agilent), followed by paired-end sequencing on HiSeq 2000 (2 × 100 cycles) (Illumina). Reads were mapped against the human reference genome sequence (NCBI, GRCh37/hg19) with the CLC Genomics Workbench v6.4 (Qiagen), followed by postmapping duplicate-read removal, coverage analysis, and quality-based variant calling. More thorough variant annotation was executed with Alamut-HT v1.1.5 and Alamut Visual v2.7 software (Interactive Biosoftware). For case 1, because we had access to parental DNA, a trio-sequencing strategy was used. Variant filtering was performed with the Ingenuity Variant Analysis platform (Qiagen). Sanger sequencing was used to confirm potentially pathogenic variants identified by WES and to perform segregation analysis. Primers were designed with primer3plus. Sequencing was performed on the ABI 3730XL DNA Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), followed by data analysis with SeqScape v2.5 (Life Technologies). Microsatellite analysis with 29 markers (deCODE, Généthon, and Marshfield) was performed to assess disease haplotypes in the three mutation–positive cases. Data analysis was performed with the GeneMapper v3.7 software (Applied Biosystems).

In silico modeling of the p.(Arg92Trp) and p.(Arg92Gln) mutations
All modeling and calculations were performed using model 1 of the NMR structure of the NR5A1 DNA binding domain (PDB code: 2fho).15 The effect of the mutations on the stability of the protein was calculated using the FoldX RepairPDB and BuildModel commands, with 20 replicate calculations.16 Models for p.(Arg92Trp) and, for comparison, p.(Arg92Gln) mutants of the NR5A1 DNA-binding domain in complex with DNA were built using the YASARA structure with the swap and optimize commands to replace R92 by a tryptophan or glutamine, followed by energy minimization with the YASARA forcefield in explicit solvent.17 The in silico binding energy between the NR5A1 LBD and the DNA fragments was calculated using the YASARA BindEnergy command.17 Models and structures were visualized via UCSF chimera.18

Plasmid construction
We constructed a GFP-tagged NR5A1 wild-type (WT) construct starting from the Gateway pcDNA-DEST47 vector (Invitrogen, Life Technologies) and a WT NR5A1 open...
NR5A1 mutation causes 46,XX (ovotesticular) DSD | BAETENS et al

The mutations undergoing study were inserted using the Q5 site-directed mutagenesis kit (New England Biolabs), and mutation-specific primers were designed with the NEBaseChanger software (New England Biolabs). Mutated plasmids were transformed in One Shot TOP10 competent cells (Invitrogen, Life Technologies) and subsequently isolated with the NucleoBond Xtra Midi/Maxi kit (Macherey-Nagel). The entire NR5A1 insert and the surrounding backbone were sequenced and selected plasmids were grown to obtain larger quantities.

Luciferase assays
Luciferase assays were performed in two cell lines: HeLa and KGN. Cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. One day before transfection, 5,000 cells/well were seeded in a 96-well plate. Each experiment was performed in six independent replicates. Cells were transfected with 187 ng of a DNA mixture using calcium phosphate transfection, after which they were washed with phosphate-buffered saline and ethylene glycol tetra-acetic acid to remove remaining precipitates. After 48 hours, cells were washed with phosphate-buffered saline again before...
RESULTS

Novel and recurrent missense mutation in NR5A1 in three patients with 46,XX (Ovo)Testicular DSD

Eleven unrelated patients and two sisters with 46,XX ovo-testicular or testicular DSD were included in this study. Nine underwent WES and four underwent resequencing of the coding region of NR5A1 because they were included at a later time. When exome data were available, all variants were filtered against a list of known 46,XX and 46,XY DSD genes (Supplementary Data online, Supplementary Table S2 online), annotated, and manually curated. All variants in DSD genes, identified in cases 1–9, are listed in Supplementary Data online and Supplementary Tables S3–S11 online. We identified a heterozygous missense variant in exon 4 of NR5A1 c.274G>T p.(Arg92Trp) in cases 1–3. Prediction programs SIFT, Polyphen-2, and MutationTaster suggest a possible effect on protein function. The physicochemical distance between Arg and Trp is moderate (Grantham score of 101) and the variant is absent in genomic databases such as dbSNP,
NR5A1 mutation causes 46,XX (ovo)testicular DSD | BAETENS et al

The NR5A1 mutation causes 46,XX (ovo)testicular DSD, and the authors investigated this mutation in three families. They found that the affected amino acid, R92, is located in a region C-terminal to the classical DBD with two zinc (Zn) fingers. This additional domain is found in family members of NR5A1, including NGFI-B, and was called the A-box or Ftz-F1 box. This A-box domain has been implicated in interactions with the minor groove of the target DNA. Prior to structure determination, binding studies with NR5A1 and NGFI-B mutants showed that this interaction is important for their binding specificity toward different DNA targets. For NR5A1, this specificity is determined by amino acids 91–93, which are predicted to interact with three specific basepairs of the classical half-site bound by the Zn fingers. The results of this study are in line with those for the later NMR structure of the NR5A1 DBD bound to a DNA fragment of the inhibin-alpha subunit promoter. In this structure, the side chain of R92 inserts deeply into the minor groove, and its guanidine head group binds to two of the three basepairs at the 5′ site of classical half-sites. Mutation R92A abolished DNA-binding and signaling via the inhibin-alpha subunit promoter in this study by removing the interaction with the basepairs of the minor groove. The p.(Arg92Trp) mutation is not allowed in the structure without gross structural rearrangements; the W side chain cannot insert into the minor groove and cannot be accommodated in the current DNA-bound structure without inducing steric clashes.

**Protein structure modeling of NR5A1 p.(Arg92Trp) and p.(Arg92Gln)**

We performed protein structural modeling, starting from a model of NR5A1 bound to the α-inhibin promoter, for the following mutations: p.(Arg92Trp) found here in the three cases with 46,XX DSD and p.(Arg92Gln) previously found in 46,XY DSD. FoldX calculations predict that the mutations do not affect the stability of the isolated NR5A1 DNA-binding domain. In the models of mutants bound to DNA, the tryptophan and glutamine side chains do not penetrate as deeply into the minor groove as does the wild-type arginine side chain. Although the Arg92 side chain makes a hydrogen bond with a deoxyribonucleosine base, no hydrogen bonds are formed by the mutant side chains. The (enthalpic) in silico binding energy for interaction of the DNA-binding domain of NR5A1 with the DNA segment is 9% lower for both mutants. This suggests that both mutations can affect the interaction of NR5A1 with its response elements, although effects may be variable and specific for different response elements (Figure 2).
Transcriptional activity and subcellular localization of NR5A1 mutants p.(Arg92Trp) and p.(Arg92Gln)

We assessed the impact of following NR5A1 mutations on transcriptional activity and subcellular localization in different cellular systems: c.274C>T p.(Arg92Trp) and the previously reported adjacent mutation c.275G>A p.(Arg92Gln). For the latter mutation, partial loss of DNA-binding and transcriptional activity have previously been shown.24 We cotransfected WT or mutagenized NR5A1 plasmids with different NR5A1 responsive promoters (TESCO, SOX9, AMH, CYP11B1) in different cell lines (HeLa, KGN). These experiments were repeated with FOXL2 cotransfection because it was shown previously that FOXL2 inhibits NR5A1-mediated SOX9 expression during female development.25 The results of these assays were inconclusive, and only the results for the TESCO-luc constructs are shown (Supplementary Data online, Supplementary Figure S1 online). These experiments reproduced the previously described lower transcriptional activity of the c.275G>A p.(Arg92Gln) variant. The c.274C>T p.(Arg92Trp) variant, however, led to decreased activity in HeLa cells but not in KGN cells, showing that the transcriptional activity of a variant might be influenced by the cellular environment. Subcellular localization assays in HeLa cells showed nuclear expression without aggregate formation for both WT and mutant NR5A1 (Supplementary Data online, Supplementary Figure S2 online).

Transcriptome analysis in patients' lymphocytes

RNA-seq generated an average of 39 million reads per sample. Differential expression analysis with default settings showed a total of 1,420 genes with significant differential expression (corrected P-value < 0.05) when comparing the patient and control groups (Supplementary Data online, Supplementary Table S17 online). This gene set was filtered against a list of DSD-associated genes (Supplementary Data, Supplementary Table S2), the coverage of which was low in general. This filtering resulted in three (LEPR, MAMLD1, and IRF2BP1) genes with differential expression (Supplementary Data, Supplementary Table S18).
NR5A1 mutation causes 46,XX (ovo)testicular DSD | BAETENS et al

MAMLD1, a direct target gene of NR5A1 (ref. 26) that was previously reported to be involved in 46,XY DSD (ref. 27), was upregulated (log 2-fold change = 1.637, \( P = 0.03 \)) in patients compared with controls. BioGPS, an online available expression database, shows expression in both testis and ovaries in mice. LEPR and IREF2BPL (EAP1) are genes involved in hypogonadotropic hypogonadism, which we considered not directly relevant in the context of the phenotypes observed here.

**Immunostaining of SOX9, FOXL2, and DDX4 on patients’ gonads**

Gonadal biopsy specimens from cases 1 and 3 and a gonadectomy specimen from case 2 were available. In case 1, a dysgenetic testis with scarce germ cells, as identified by DDX4 immunostaining, was found. Biopsy specimens for case 3 revealed bilateral immature testes with Sertoli cell–only tubules (i.e., no germ cells present). Case 2 had bilateral ovotestes, including the presence of primordial and primary follicles in the ovarian part, located at the pole of the gonad, and next to immature seminiferous tubules without germ cells, located in the central testicular part of both gonads. Immunohistochemistry with SOX9 and FOXL2 antibodies, identifying Sertoli and granulosa cell differentiation, respectively, revealed exclusive SOX9 expression in testicular parts and exclusive FOXL2 expression in ovarian parts of case 2 (Figure 3). Hematoxylin and eosin staining of ovarian tissue, removed after ovarian torsion at age 41 years in the mother of case 2, was unremarkable and showed an aging ovary with some residual primordial follicles and stromal tissue (Supplementary Data online, Supplementary Figure S3 online).

**DISCUSSION**

A few NR5A1 mutations have been identified in females with 46,XY complete gonadal dysgenesis and adrenal failure. Interestingly, one of these was an adjacent homozygous mutation, c.275G>A p.(Arg92Gln), in the same codon as the c.274C>T p.(Arg92Trp) mutation identified here. Lower transcriptional activity was shown for p.(Arg92Gln), suggesting a loss-of-function effect. This mutation was also recently identified in a 46,XX female with adrenal insufficiency but no

Figure 4 Schematic overview of the sex development gene regulation network. Blue: testis-promoting activity of NR5A1 (ref. 1). Orange: counteractive connections to suppress the opposite pathway. Green: ovary-promoting activity of NR5A1 as hypothesized here and supported by other works.45,46 (a) General scheme: NR5A1 is known to initiate the male developmental pathway through upregulation of SOX9 (synergistically with SRY). SOX9 then maintains its own expression via Fgf9 signaling. In the female embryo, in the absence of SRY, NR5A1 induces WNT4 and RPSO1 expression as shown by Combes, leading to upregulation of FOXL2 and other ovary-specific genes. High FOXL2 expression results in stable repression of SOX9 and, possibly, NR5A1, and hence the male pathway. (b) Possible mechanism by which c.274C>T leads to ovotesticular disorders of sex development. We hypothesize that this novel mutation affects the activation of female-specific genes such as WNT4/β-catenin (as indicated by the dotted lines), leading to decreased FOXL2 expression. In this way, FOXL2 can no longer prosecute its pro-ovarian functions and, at the same time, male-promoting genes escape firm suppression, ultimately resulting in NR5A1-mediated and/or independent SOX9 upregulation and enhancement of testicular differentiation.
gonadal phenotype. Subsequently, numerous NR5A1 mutations and copy number variations have been associated with isolated 46,XY gonadal dysgenesis, 46,XY undervirilization, and male infertility. In 46,XX individuals, these loss-of-function mutations may cause primary ovarian insufficiency (POI). This broad range of phenotypes emphasizes that correct NR5A1 functioning is essential for both male and female gonadal development and maintenance.

Few data exist regarding the NR5A1 expression pattern in human female and male fetuses. In humans, NR5A1 is upregulated in the undifferentiated and stage gonadal expression is maintained during the entire period of fetal development in both male and female fetuses, whereas in rodents it has been shown that Nr5a1 expression decreases in the ovary as sex differentiation progresses. Indeed, NR5A1 plays an important role in multiple stages of gonadal development because it is a key regulator of sex determination via SOX9 upregulation during male development and of sex differentiation in both sexes via upregulation of AMH and the different steroidogenic enzymes. Both male and female Nr5a1 knockout (KO) mice lack gonadal and adrenal development. Recently, a CRISPR/Cas9 amino acid substituted mouse model was generated for p.(Arg92Trp); however, no phenotypic data were provided.

Here, the NR5A1 mutation p.(Arg92Trp) was found in three unrelated patients with 46,XX (ovo)testicular DSD. A potential founder effect was suggested by haplotype analysis. The mutation was found in XX unaffected individuals in each of the three families, suggesting incomplete penetrance, as has been observed in other NR5A1-associated phenotypes and in other large families with 46,XX (ovo)testicular DSD. In a 46,XY DSD mouse model (B6 XY pos), incomplete penetrance has been shown to result from differences in spatiotemporal expression. Of note, the geographical distribution of testicular and ovarian regions in the ovotestes of case 2 supports the hypothesis that p.(Arg92Trp) leads to more stable binding of NR5A1 to the SOX9 promoter, thereby directly inhibiting FOXL2-mediated repression of SOX9, as described by Uhlenhaut et al., which is not supported by the cellular transactivation assays performed. This is possibly because the adult cell lines that were used are not representative for early stages of gonadal development. In addition, transcriptome analysis of patient-derived lymphocytes did not reveal an upregulation of early male-specific genes. However, it did show upregulation of another NR5A1 target, MAML1.

Two animal models support this hypothesis. It was shown very recently that CRISPR/Cas9-induced Nr5a1 mutations cause female-to-male sex reversal in the XX Nile Tilapia. Nr5a1 depletion leads to reduced Cyp19A1 expression and low estradiol levels. Because ovarian differentiation in fish is mediated by estradiol, the lack of sex steroid production here was proposed as the possible trigger for changing the initial gonadal fate. Temporal expression studies of Nr5a1 and Foxl2 in this model underscore the critical importance of threshold levels of Foxl2 to downregulate Nr5a1 in the XX gonad. A second animal model is the polled intersex syndrome (PIS) goat, which is characterized by absence of horns in heterozygous and homozygous animals and female-to-male sex reversal in homozygous XX animals. This phenotype is caused by the 11.7-kb regulatory deletion that is located 300 kb upstream of the goat FOXL2 gene. Knockout experiments have shown that FOXL2 depletion leads to reduced CYP19A1 expression and that it is responsible for inhibiting ovarian development similarly to the fish model. However, if testis formation in this species is, similarly to the Nile Tilapia, mediated by reduced Nr5a1 activity, then its influence on the female gonadal pathway remains to be elucidated.

These two alternative hypotheses may explain the different phenotypes observed for the two adjacent NR5A1 mutations. Mutation c.275G>A (p.Arg92Gln) has a direct negative influence on SOX9 expression and leads to aberrant male gonadal development and adrenal failure. Variant p.Arg92Trp has no direct effect on SOX9 expression but is hypothesized to interfere with hitherto unrecognized female-promoting activity of NR5A1, ultimately resulting in loss of stable SOX9 repression.
by FOXL2. Both mutations are located in the same codon that is part of the Ftz-F1 box, which is important for DNA-binding specificity. The newly introduced amino acid might determine which NR5A1 targets are affected by the mutation and which pathways are perturbed. Structural models show that both mutations have problems entering the DNA minor groove, although they cannot predict which targets will be affected. Our results suggest that temporal, spatial, or quantitative changes disturbing the balance between the male and female pathways in the XX gonad may shift the ovarian developmental program to the testicular pathway, probably through autocrine and paracrine reinforcing signals \(^1\) (Figure 4). The exact mechanisms behind these processes need to be substantiated by additional experimental work.

Conclusion

Taking these findings together, we propose NR5A1 as a novel disease gene for 46,XX (ovo)testicular DSD. Our study suggests SRY-independent SOX9 expression at the gonadal (testicular) level, most likely not through the canonical NR5A1-SOX9 interaction, but rather through downregulation of the pr-ovarian Wnt4/b-catenin pathways, thus tipping the balance toward male development. In addition, we demonstrated that testicular and ovotesticular DSD may represent different phenotypes resulting from a common cause. We conclude that NR5A1, a well-established gene for male gonadal development, is also involved in correct female gonadal development, the exact molecular mechanisms of which are still elusive.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We are very grateful to the families who participated in this study. This work was supported by a grant from the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., by Belspo IAP project P7/43 (Belgian Medical Genomics Initiative: BeMGI) to E.D.B., by the Hercules Foundation (AUGE/13/023 to E.D.B.), by a grant from the Ghent University Special Research Fund (BOF Starting Grant) to M.C., and by grant G006713N from the Research Foundation Flanders (FWO) to E.D.B. and M.C. E.D.B. and M.C. are Senior Clinical Investigators of the FWO. F.C. is senior postdoctoral fellow of the FWO. F.C. is also involved in correct female gonadal development, the familial true hermaphroditism with partial duplication of chromosome 22 and without SRY. Am J Med Genet 1999;85:2–4.

Polanco JC, Wilhelmsen TM, Barnes J, et al. SRY-negative true hermaphroditism with partial duplication of chromosome 22 and without SRY. Am J Med Genet 1999;85:2–4.

POLITIC et al

ORIGINAL RESEARCH ARTICLE

Benko S, Gordon CT, Mallet D, et al. Disruption of a long distance regulatory region upstream of SOX9 in isolated disorders of sex development. J Med Genet 2011;48:825–830.

Sutton E, Hughes J, White S, et al. Identification of SRYX3 as an XX male sex reversal gene in mice and humans. J Clin Invest 2011;121:328–341.

Haines B, Hughes J, Corbett M. Intersomonal insertion insertion at Xp26.3 alters SOX9 expression in an individual with XX male sex reversal. J Clin Endocrinol Metab 2015;100:E815–E820.

Unterrung V, Perera EM, Bao Y, et al. 46,XX sex reversal with partial duplication of chromosome 22 and without SRY. Am J Med Genet A 2004;127A: 149–151.

Aleck KA, Argueso L, Stone J, Hackel JL, Erickson RP. True hermaphroditism with partial duplication of chromosome 22 and without SRY. Am J Med Genet 1999;85:2–4.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We are very grateful to the families who participated in this study. This work was supported by a grant from the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., by Belspo IAP project P7/43 (Belgian Medical Genomics Initiative: BeMGI) to E.D.B., by the Hercules Foundation (AUGE/13/023 to E.D.B.), by a grant from the Ghent University Special Research Fund (BOF Starting Grant) to M.C., and by grant G006713N from the Research Foundation Flanders (FWO) to E.D.B. and M.C. E.D.B. and M.C. are Senior Clinical Investigators of the FWO. F.C. is senior postdoctoral fellow of the FWO. F.C. is also involved in correct female gonadal development, the exact molecular mechanisms of which are still elusive.

by FOXL2. Both mutations are located in the same codon that is part of the Ftz-F1 box, which is important for DNA-binding specificity. The newly introduced amino acid might determine which NR5A1 targets are affected by the mutation and which pathways are perturbed. Structural models show that both mutations have problems entering the DNA minor groove, although they cannot predict which targets will be affected. Our results suggest that temporal, spatial, or quantitative changes disturbing the balance between the male and female pathways in the XX gonad may shift the ovarian developmental program to the testicular pathway, probably through autocrine and paracrine reinforcing signals (Figure 4). The exact mechanisms behind these processes need to be substantiated by additional experimental work.

Conclusion

Taking these findings together, we propose NR5A1 as a novel disease gene for 46,XX (ovo)testicular DSD. Our study suggests SRY-independent SOX9 expression at the gonadal (testicular) level, most likely not through the canonical NR5A1-SOX9 interaction, but rather through downregulation of the pr-ovarian Wnt4/b-catenin pathways, thus tipping the balance toward male development. In addition, we demonstrated that testicular and ovotesticular DSD may represent different phenotypes resulting from a common cause. We conclude that NR5A1, a well-established gene for male gonadal development, is also involved in correct female gonadal development, the exact molecular mechanisms of which are still elusive.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We are very grateful to the families who participated in this study. This work was supported by a grant from the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., by Belspo IAP project P7/43 (Belgian Medical Genomics Initiative: BeMGI) to E.D.B., by the Hercules Foundation (AUGE/13/023 to E.D.B.), by a grant from the Ghent University Special Research Fund (BOF Starting Grant) to M.C., and by grant G006713N from the Research Foundation Flanders (FWO) to E.D.B. and M.C. E.D.B. and M.C. are Senior Clinical Investigators of the FWO. F.C. is senior postdoctoral fellow of the FWO. F.C. is also involved in correct female gonadal development, the exact molecular mechanisms of which are still elusive.

REFERENCES

1. Ono M, Harley VR. Disorders of sex development: new genes, new concepts. Nat Rev Endocrinol 2013;9:79–91.

2. Li TF, Wu QY, Zhang C, et al. 46,XX testicular disorder of sexual development with SRY-negative caused by some unidentified mechanisms: a case report and review of the literature. BMC Urol 2014;14:104.

3. Huang B, Wang S, Ning Y, Lamb AN, Barley J. Autosomal XX sex reversal caused by duplication of SOX9. Am J Med Genet 1999;87:349–352.
Five novel mutations in steroidogenic factor 1 (SF1, NR5A1) in 46,XY patients with severe underandrogenization but without adrenal insufficiency. Hum Mutat 2008;29:59–64.

Köhler B, Lin L, Ferraz-de-Souza B, et al. Five novel mutations in steroidogenic factor 1 (SF1, NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 2009;161:237–242.

Köhler B, Lin L, Mazen I, et al. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, NR5A1) in 46,XY patients with severe underandrogenization but without adrenal insufficiency. Hum Mutat 2008;29:59–64.

Barbaro M, Cools M, Looijenga LH, Drop SL, Wedell A. Partial deletion of the NR5A1 (SF1) gene detected by synthetic probe MLPA in a patient with XY gonadal disorder of sex development. Sex Dev 2011;5:181–187.

Bashamboo A, Ferraz-de-Souza B, Lourenço D, et al. Human male infertility associated with mutations in NR5A1 encoding steroidogenic factor 1. Am J Hum Genet 2010;87:505–512.

Köhler B, Lin L, Mazen I, et al. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 2009;161:237–242.

Köhler B, Lin L, Mazen I, et al. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 2009;161:237–242.

Köhler B, Lin L, Mazen I, et al. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 2009;161:237–242.