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Human Male Infertility Associated with Mutations in *NR5A1* Encoding Steroidogenic Factor 1

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One in seven couples worldwide are infertile, and male factor infertility accounts for approximately 30%–50% of these cases. Although many genes are known to be essential for gametogenesis, there are surprisingly few monogenic mutations that have been conclusively demonstrated to cause human spermatogenic failure. A nuclear receptor, NR5A1 (also called steroidogenic factor 1), is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis, and it is expressed in the steroidogenic tissue of the developing and adult human gonad. Mutations of *NR5A1* have been reported in 46,XY disorders of sex development and in 46,XX primary ovarian insufficiency. To test the hypothesis that mutations in *NR5A1* cause male infertility, we sequenced *NR5A1* in 315 men with idiopathic spermatogenic failure. We identified seven men with severe spermatogenic failure who carried missense mutations in *NR5A1*. Functional studies indicated that these mutations impaired NR5A1 transactivational activity. We did not observe these mutations in more than 4000 control alleles, including the entire coding sequence of 359 normospermic men and 370 fertile male controls. *NR5A1* mutations are found in approximately 4% of men with otherwise unexplained severe spermatogenic failure.

Introduction

It is estimated that one in seven couples worldwide have problems conceiving.[1] In recent years there has been increasing concern about a possible decline in reproductive health, and this trend is paralleled by an increasing demand for infertility treatments. As many as 8% of children in some Western countries are born as a result of assisted reproductive techniques.[2] Sperm counts in several European countries are declining, and in Denmark 20% of healthy young adult males have sperm concentrations below the World Health Organization reference level of 20×10^6 sperm/ml.[1,3] In the majority of cases, the underlying cause of male infertility is unknown. Familial clustering of male subfertility as well as families with multiple infertile or subfertile men, in whom an autosomal-recessive or -dominant mutation with sex-limited expression is likely to be present, indicates a genetic contribution to spermatogenic failure.[4–6] A chromosomal anomaly is carried by 5% of all infertile men (such as 47,XXY Klinefelter syndrome), and microdeletions of the long arm of the Y chromosome (MIM 415000) are present in 10% of azoospermic or severely oligozoospermic ($< 1 \times 10^6$ sperm/ml) men.[7] Although rodent studies indicate that multiple genes have the potential to cause male infertility, only a few single-gene defects that cause male infertility have been identified in humans. These include *AURKC* (MIM 603495) mutations associated with large-headed, multiflagellar polyploid spermatozoa (MIM 243060), *SPATA16* (MIM 609856) mutations associated with globozoospermia (MIM 102530), *CATSPER1* (MIM 606389) mutations associated with recessive male infertility (MIM 612997), and mutations of the dynein genes that encode proteins of the axonemal dynein cluster (*DNAH1* [MIM 603332], *DNAH5* [MIM 603335], *DNAH11* [MIM 603339]) and are associated with asthenozoospermia.[8] However, the collective prevalence of these mutations is extremely low.

NR5A1 (MIM 184757), a member of the nuclear receptor superfamily, is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis.[9,10] NR5A1, also called steroidogenic factor-1, consists of a DNA-binding domain (DBD) including two zinc fingers, a flexible hinge region, a ligand-binding domain (LBD), and two activation function domains: AF-1 and AF-2.[11–13] NR5A1 binds DNA as a monomer, and it is expressed in Sertoli and Leydig cells of the developing testis and in Sertoli cells of the prepubertal and adult testis, as well as in multiple cell types in the fetal, postnatal, prepubertal, and mature ovary.[14–16] In mammalian testis determination and differentiation, NR5A1 is a positive regulator of SOX9 (Sry-box 9) and Anti-Müllerian Hormone (AMH).[17,18] NR5A1 also modulates the expression of many factors involved in cholesterol mobilization and steroid hormone biosynthesis, including HMG-CoA synthase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), and several cytochrome P450 steroid

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hydroxylase (CYP) enzymes.[17] Consistent with its key role in gonadal development, *NR5A1* mutations are associated with a wide spectrum of phenotypes, including 46,XY partial and complete gonadal dysgenesis with or without adrenal failure (MIM 612965), penoscrotal hypospadias, micropenis with anorchidia, and 46,XX primary ovarian insufficiency (POI [MIM 612964]).[17,19]

Here, we demonstrate that heterozygous mutations in *NR5A1* are also associated with severe spermatogenic failure in otherwise healthy men. In an analysis of 315 men seeking infertility treatment because of spermatogenic failure, we identified heterozygous missense mutations in seven men, each mutation located in the hinge region and proximal LBD of the protein. Each of the mutant proteins fails to transactivate gonadal promoters optimally. Our data increase the spectrum of phenotypes that are associated with mutations in *NR5A1*.

Subjects and Methods

Patient and Control Populations

The study was approved by the Institut Pasteur institutional review board (RBM 2003/8). We obtained written informed consent from all patients, family members, and control subjects who participated in the study. A total of 315 men who had unexplained reduced sperm counts and were seeking infertility treatment were included in this study. All men were recruited from one infertility clinic in Paris. The men were of mixed ancestry, and they are representative of the local Parisian population. Patient ancestry was determined by self reporting, based on responses to a personal questionnaire, which asked questions pertaining to the birthplace, languages, and ethnicity of the participants, their parents, and their grandparents. Infertile men with known causes of infertility, including chromosome anomalies, Y chromosome microdeletions, cryptorchidism, hypospadias, occupational hazards, varicocele, and lifestyle factors, were excluded from this study. Control samples were obtained from the HGDP-CEPH panel, comprising 1064 DNA samples from 52 worldwide populations. Additional control samples consisted of 140 French men, 89 men of West African origin (kindly provided by Dr. Anavaj Sakuntabhai of the Institut Pasteur, Paris), and 96 men of North African origin. Although these men are healthy, their fertility and semen quality is unknown. To investigate the degree of rare genetic variation in *NR5A1*, we sequenced the entire open reading frame of *NR5A1* in DNA from a panel of 370 fertile men (father of at least two children) and 359 men with normal semen parameters (European descent n = 331, North African descent n = 140, West African descent n = 63, Indian descent n = 156, East Asian descent n = 30, other descent n = 9).

Mutational Analysis of *NR5A1*

The coding exons of *NR5A1* (exons 2–7; NM_004959.4) were amplified from DNA extracted via conventional techniques from peripheral-blood lymphocytes of each individual and sequenced in accordance with protocols described elsewhere.[19]

Site-Directed Mutagenesis

NR5A1 expression vectors containing the p.Pro131Leu, p.Arg191Cys, p.Asp238Asn, and p.Gly212Ser variants were generated by site-directed mutagenesis (QuikChange, Stratagene) with the use of wild-type (WT) human *NR5A1* cDNA in a pCMX expression vector as a template.[19] The entire coding sequence of all mutant plasmids was confirmed by direct sequencing prior to functional studies.

Transient Gene Expression Assays

Transient gene expression assays for the assessment of NR5A1 function were performed in 96-well plates (TPP) with the use of either human embryonic kidney (HEK) 293T cells or a mouse embryonic stem cell line (E14), Fugene 6 transfection reagent (Roche no. 1 814 443), and a Dual-Luciferase reporter assay system (Promega) with pRLSV40 Renilla luciferase (Promega) expression as a marker of transfection efficiency. pCMX_WT or mutant NR5A1 expression vectors (10 ng/well) were cotransfected into HEK293T cells with reporters containing NR5A1 (SF1) responsive minimal promoters (murine *Cyp11a1*, human *AMH*) (10 ng/well).[20,21] Cells were lysed 48 hr later, and luciferase assays were performed with the use of a FLUOstar Optima fluorescence microplate reader (BMG Labtech). All data were standardized for Renilla activity. Results are shown as the mean ± SEM of three independent experiments, each performed in triplicate. A previously described inactivating mutation of NR5A1, p.Gly35Glu, was included as control in the transactivation studies.[21]

Cellular Localization Studies

WT *NR5A1* cDNA was cloned into a pAcGFP-C1 vector (Clontech) to allow expression of GFP-tagged NR5A1. *NR5A1* mutations were introduced by site-directed mutagenesis. Plasmids (0.8 μg/well) were transfected into tsa201 cells with the use of Lipofectamine 2000 (Invitrogen), and images were obtained 24 hr later with a Zeiss Axioskop microscope and camera.

In Vitro Protein Expression

An in vitro rabbit reticulocyte-coupled transcription/translation system (TNT Quick Coupled Transcription/Translation System, Promega) was used to express proteins from the vector constructs. The reactions were performed according to the manufacturer's instructions. In brief, biotin-labeled protein was expressed by incubation of 1 μg of vector DNA with reticulocyte lysate, amino acid mixture, RNasin, T7 RNA polymerase, and Transcend Biotin-Lysyl-tRNA (Promega) in a final volume of 25 μl at 30°C for 90 min.

Table 1. Mutations in *NR5A1* Associated with Spermatogenic Failure

Patient	Age at Investigation	Ethnic Origin	Karyotype	*NR5A1* Mutation	Sperm Count (10^6/ml) N: > 20 × 10^6/ml	FSH (IU/l) N: 1.0–10.5 IU/l	LH (IU/l) N: 0.7–8.0 IU/l	Testosterone (ng/ml) N: 3.0–10 ng/ml	Inhibin B (pg/ml) N: 80–400 pg/ml
1	42	Congolese	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	0	72	34.3	0.49	<15
2	37	Congolese	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	0	NA	NA	NA	NA
3	29 and 31[b]	Tunisian	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	12 and 6[c]	5.1	4.3	5	74
4	41	Sri Lankan[d]	46,XY	p.Pro131Leu (c.392C>T)	0	NA	NA	NA	NA
5	25	Congolese	46XY	p.Arg191Cys (c.571C>T)	0.3	18.8	10.7	5.7	<15
6	37	French-Vietnamese	46XY	p.Gly212Ser (c.634G>A)	0.8	NA	NA	NA	NA
7	41	Egyptian	46,XY	p.Asp238Asn (c.712G>A)	0.7	15.1	6	3	31

NA, not available.
[a] Mutation was previously reported as associated with POI.
[b] Patient was evaluated over a 2 yr period.
[c] Semen quality decreased over a 2 yr period. There was also a reduction in sperm motility and viability.
[d] Mutation also observed in a woman with POI who is of Tamil origin (unpublished data).

Sumoylation of Mutated Proteins

In vitro sumoylation assays were carried out by incubating in vitro translated pCMXSF1 or p.Asp238Asn vectors with recombinant Aos1/Uba2 (370 nM), Ubc9 (630 nM), and SUMO (7 μM) in 30 mM Tris, 5 mM ATP, 10 mM $MgCl_2$, pH 7.5, at 33°C as previously described.[22]

Results

NR5A1 Mutations Were Identified in Infertile Men

In a screen of 315 men with unexplained spermatogenic failure who sought infertility treatment, we found seven heterozygous mutations in *NR5A1* by direct sequencing (Table 1). The seven men carrying *NR5A1* mutations did not report any other members of the family with phenotypes known to be associated with *NR5A1* mutations, such as POI or 46,XY disorder of sex development (DSD), and there was no evidence of undervirilization, nor were there signs of adrenal insufficiency. No other family members were available for genetic analysis, so it is unknown whether the mutations are de novo. However, three men of African origin carried a double *NR5A1* mutation (p.Gly123Ala/p.Pro129Leu; NP_004950) that we have previously reported as being associated with POI in a girl of African origin, suggesting that this is probably a founder mutation.[19] One man, who carried the p.Gly123Ala/p.Pro129Leu double mutation, had a progressive loss of germ cell quantity and quality over a 2 yr period (Table 1). With one exception, *NR5A1* mutations were associated with severe spermatogenic failure (Table 2). We did not observe mutations in men with mild oligozoospermia. These mutations were not observed in over 2100 control samples (4200 alleles), and no rare allelic variants were found after analysis of the entire coding region of *NR5A1* in 370 fertile (father of at least two children) or 359 normospermic men (Table 2). This indicates that these mutations are pathogenic. All mutations fall within the evolutionarily conserved hinge region (amino acids 95–225), or the proximal portion of the LBD of NR5A1 (Figure 1).

NR5A1 Mutations Are Associated with Altered Hormonal Profile and Gonad Histology

Hormonal data were available for four of the men carrying *NR5A1* mutations (Table 1). Testosterone levels were at the lower limit of the normal range in subject 7 and below the normal range in subject 1. Serum levels of inhibin B, a marker of spermatogenesis and a predictor of the presence of testicular sperm in men with nonobstructive

Table 2. Frequency of *NR5A1* Mutations and Associated Phenotypes

Phenotype	No. of Individuals	No. of Individuals with Mutation in *NR5A1*
Azoospermia or cryptozoospermia	103	4 (3.9%)
Severe oligozoospermia or OATs < 1 × 10^6/ml	46	2 (4.3%)
Moderate oligozoospermia or OATs 1–10 × 10^6/ml	50	1 (2%)
Mild oligozoospermia or OATs 10–20 × 10^6/ml	116	0
Fertile[a]	370	0
Normospermic[a]	359	0

[a] The entire open reading frame of *NR5A1* was sequenced in each individual.

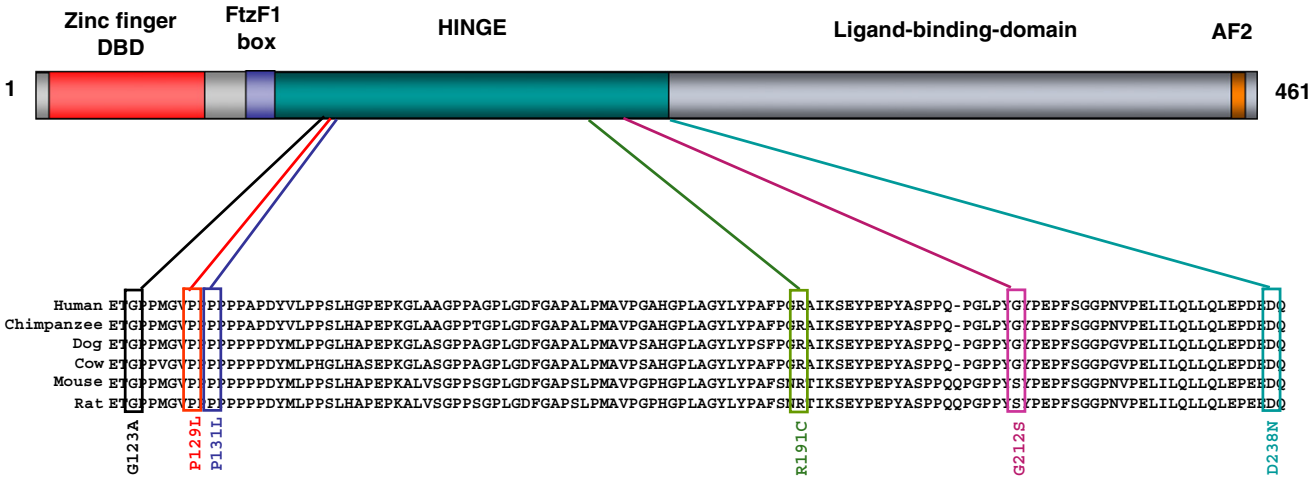

Figure 1. Distribution of NR5A1 Mutations Associated with Spermatogenic Failure in Relation to the Protein
The functional domains of the NR5A1 protein are shown. The DNA-binding domain containing two zinc-finger motifs is indicated. The FtzF1 box stabilizes protein binding to DNA. The hinge region is important for stabilizing the ligand-binding domain and interacts with other proteins that control NR5A1 transcriptional activity. The AF2 domain recruits cofactors necessary for NR5A1 transactivating activity. The position of the amino acid change and its evolutionary conservation are shown for each of the mutations identified.

azoospermia, were low in all four men. Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were elevated above the normal values in cases 5 and 1, whereas case 7 had elevated serum FSH and LH levels within the normal range. Gonadal histology was available for case 1, who carries the p.Gly123Ala/p.Pro129Leu double mutation (Figure 2). This showed a hypoplastic testis with few germ cells and areas of marked fibrosis and hyalinization.

NR5A1 Mutations Do Not Affect Nuclear Localization but Alter the Transactivation Ability of the Protein

To assess the impact of the NR5A1 mutations on nuclear localization, we generated WT and mutant GFP-NR5A1 constructs by cloning WT *NR5A1* cDNA in frame into a pAcGFP-C1 vector to produce a fusion protein of NR5A1 with a monomeric green fluorescent protein (GFP) tag at its amino-terminal end. Mutant pAcGFP-C1-NR5A1 vectors were generated by site-directed mutagenesis, with the WT construct used as a template. The cellular localization of both WT and mutant GFP-NR5A1 fusion proteins (green), generated and expressed in tsa201 cells with the use of the pAcGFP-C1 vector, showed strong nuclear localization with relative nucleolar exclusion and very occasional nuclear subfoci (Figure 3).

To assess the functional properties of NR5A1, we performed site-directed mutagenesis by using WT human *NR5A1* cDNA in a pCMX expression vector as a template.

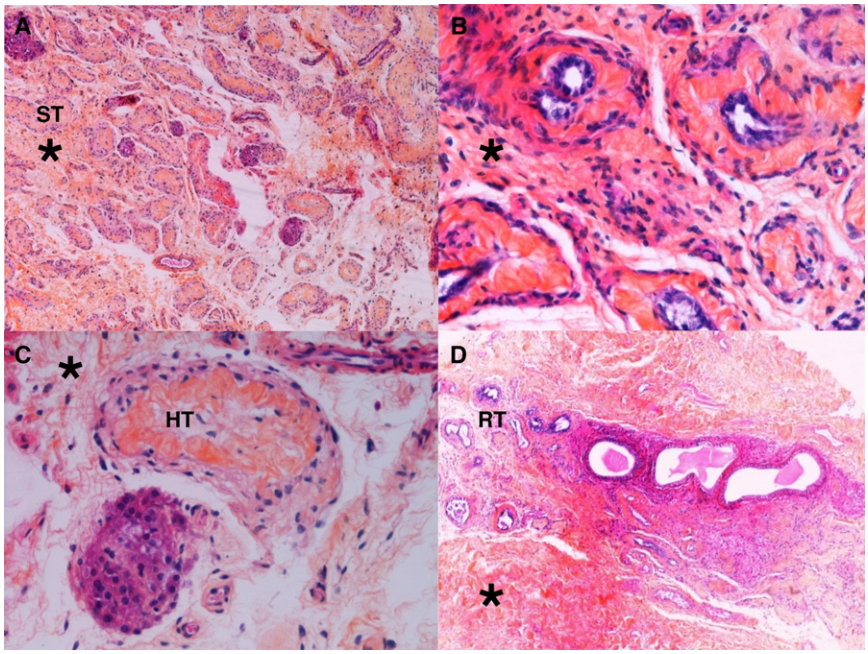

Figure 2. Gonadal Histology of Subject 1
Photomicrographs demonstrating abnormal testicular histology showing areas of interstitial fibrosis (A–D) and hyalinization (C), with scattered residual abnormal seminiferous tubules containing occasional germ cells but no normal spermatogenesis. Within the fibrous areas, residual tubular structures are present (D). No normal testicular tissue is present. (ST, seminiferous tubules; *, interstitial fibrosis; HT, hyalinised tubule; RT, residual tubular structures).

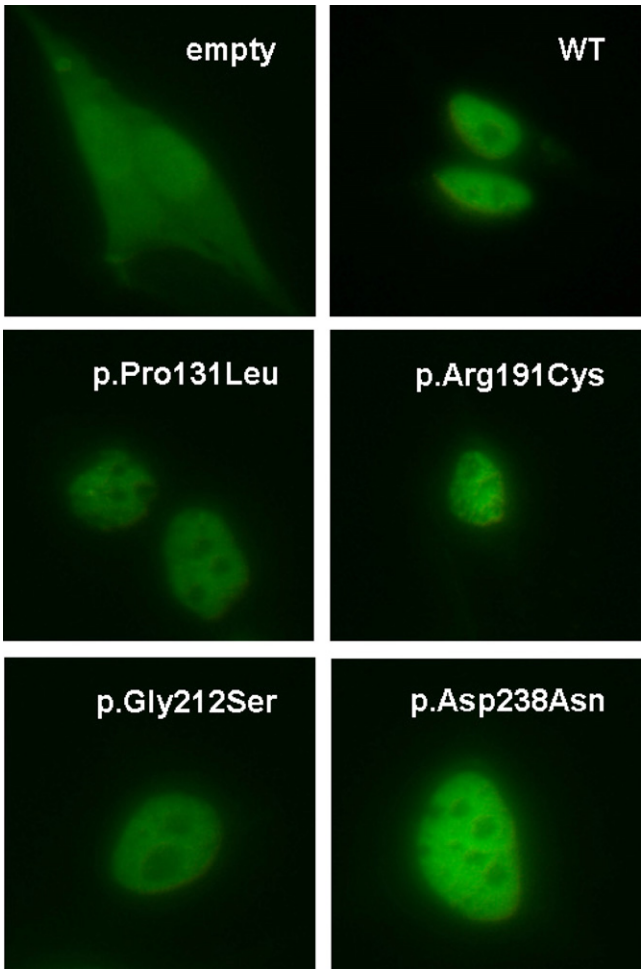

Figure 3. Cellular Localization of NR5A1 Mutants
Cellular localization of GFP-SF1 fusion proteins (green), generated and expressed in tsa201 cells with the use of a pAcGFP-C1 vector. WT NR5A1 shows strong nuclear localization, with relative nucleolar exclusion and very occasional nuclear subfoci. An expression and localization pattern similar to that of the WT was seen for all the other mutant proteins.

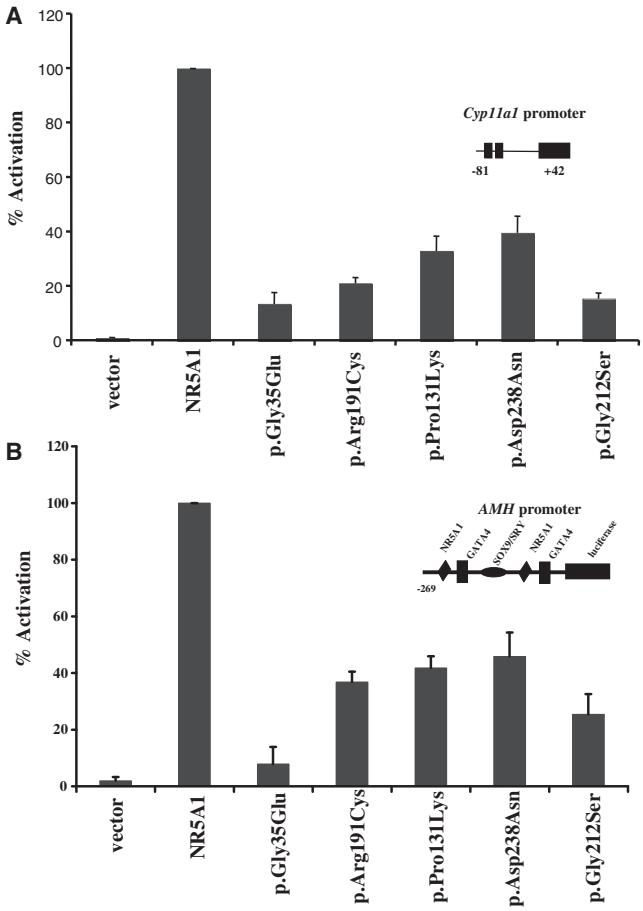

Figure 4. Assays of NR5A1 Transcriptional Activity
The transcriptional activity of WT NR5A1 and variants associated with male infertility was studied with the use of *Cyp11a1* (A) and *AMH* (B) promoters in HEK293T cells. A previously described inactivating mutation of NR5A1, p.Gly35Glu, was included as a control in the transactivation studies. Results are expressed as a percentage of WT NR5A1 activity, which is considered to be 100%. Data represent the mean of three independent experiments, each performed in triplicate. The T bars represent the SEM.

We have previously demonstrated that the NR5A1 p.Pro129Leu variant lacks transcriptional activity, and this mutant protein is associated with 46,XX POI.[19] The other mutant proteins had altered biological activity in cotransfection luciferase assays driven by NR5A1-dependent gonadal promoters. A quantitative reduction in the transactivation of both the *Cyp11a1* (encoding P450scc) promoter and the *AMH* (encoding anti-Müllerian hormone) promoter was observed in transactivation assays of the mutations p.Pro131Leu, p.Arg191Cys, p.Gly212Ser, and p.Asp238Asn (NP_004950) using HEK293T cells (Figures 4A and 4B). Similar results were obtained in transient gene expression assays using murine E14 embryonic stem cells (data available on request). These in vitro functional assays demonstrated that each mutation may lead to a functional disturbance of the NR5A1 protein and may affect the regulation of its downstream target genes during gonadal development and function.

NR5A1 Mutation p.Asp238Asn Does Not Affect Sumoylation of NR5A1

The NR5A1 p.Asp238Asn mutation was analyzed for its ability to undergo sumoylation because it lies immediately carboxy terminal to a putative SUMO-binding motif. No difference was observed between the efficiency of pCMX-SF1 and pCMX-Asp238Asn for undergoing in vitro sumoylation (data available upon request).

Discussion

In this study, we provide evidence that mutations in *NR5A1* (encoding steroidogenic factor 1) are associated with unexplained severe spermatogenetic failure in otherwise healthy men. This considerably broadens the range of phenotypes associated with mutations in *NR5A1*, which to date have been reported only in association with more severe forms of gonadal dysgenesis or with significant genital anomalies

such as penoscrotal hypospadias, anorchia, or undescended testes.[17] These data therefore support the hypothesis of Skakkebaek and coworkers that a subset of men with spermatogenic failure have a mild form of testicular dysgenesis syndrome.[1,23] Although male factor infertility is the primary presenting feature leading to medical evaluation in these cases, our data show that this subset of men with severe azoospermia may also be at risk of endocrine dysfunction and failing testosterone with increasing age. It is well established that approximately 12%–15% of men with idiopathic spermatogenic failure have reduced serum testosterone levels and elevated LH levels as compared to the normal range.[24] It has been proposed that some of these men may have mild forms of testicular dysgenesis.[24] Our data suggest that those individuals with *NR5A1* mutations may represent part of this group.

The mutations in NR5A1 reported here were all missense mutations in the hinge region or proximal ligand-binding domain of the protein. We found the p.Gly123Ala/p.Pro129Leu double mutation in three individuals of Central or North African ancestry, and we have described this change previously in a West African girl with POI, suggesting that this mutation may be present at low levels in the general population.[19] The transmission of the mutation may be explained by a progressive loss of gonadal function over time, so that fecundity is achieved in early adulthood before the development of spermatogenic failure. In this study, two men who carried this mutation presented with azoospermia at 37 and 42 yrs of age. Individual 3, who also carried this mutation, showed a progressive decline in both sperm quantity and quality over a 2 yr period, from 29 yrs to 31 yrs of age. This may represent a progressive aging phenomenon or may possibly represent different expression of the phenotype as a result of other genetic or environmental modifiers. The absence of this allelic variant in the control population suggests that this is not a frequent genetic alteration. Furthermore, the absence of any changes in *NR5A1* after direct sequencing of more than 600 fertile or normospermic men suggests that rare allelic variants in this gene are not common, other than the well-described p.Gly146Ala polymorphism (rs1110061).

A number of molecular mechanisms may explain the spermatogenic failure associated with *NR5A1* mutations. In the *Nr5a1* Leydig cell-specific knockout, mice had hypoplastic testes in which the lumens of the seminiferous tubules failed to open and spermatogonia never developed into mature sperm.[25] These mice also showed reduced expression of two key genes in testosterone biosynthesis, *Cyp11a* and *StAR*.[25] In a study of azoospermic patients, expression levels of *NR5A1* in gonadal tissue correlated positively with serum testosterone concentrations, suggesting a direct connection between these two factors.[26] Alternatively, impairment of reproductive function could result, in part at least, from anomalies of the anterior pituitary. Mice that lack *Nr5a1* specifically in their pituitary have reduced levels of LH and FSH.[27] These mice show marked hypogonadism with a reduction in testis volume, a decreased number of Leydig cells, and an absence of mature spermatids, resulting in infertility.[27] However, our data show normal or elevated serum FSH and LH levels together with a normal or low testosterone level in men carrying an *NR5A1* mutation that suggests a predominant testicular phenomenon.

We show that the NR5A1 mutants associated with male infertility show impaired activation of two of the NR5A1 target genes, *AMH* and *Cyp11a1*. Several molecular mechanisms could explain the germ cell loss associated with these mutations. The mutations fall within the hinge region (amino acids 95–225) and proximal portion of the LBD, and a number of physical interactions and functional activities have been mapped to this portion of the protein.[12] Phosphorylation of Ser 203 in the hinge region enhances the interaction of GRIP1 and SMRT with the AF1 and AF2 regions of NR5A1, whereas sumoylation of lysines within the hinge region increases interactions with DEAD box proteins and results in transcriptional repression.[28,29] NR5A1 stimulation of CYP17A1 expression is augmented by a direct physical interaction with the protein translin.[30] This interaction is mediated through amino acids 170–225 of the hinge region of NR5A1.[30] Modulation of NR5A1 transcriptional activation via the hinge region has also been described for the coactivator SRC-1, which potentiates the activity of SF-1 by utilizing the highly-conserved AF-2 hexamer at the C terminus of the protein and a proximal interaction domain at residues 226–230.[16] Finally, in vitro studies have suggested that NR5A1 receptor phosphorylation may be modulated by the herbicide atrazine, leading to disruption of NR5A1-related gene networks and potential alterations in endocrine development and function.[31] The clustering of the mutations in a specific region of the molecule could suggest a common mechanism leading to germ cell loss, or even altered sensitivity to environmental disruptors, but additional studies of larger cohorts of infertile men are required to see whether such a genotype-phenotype correlation is robust.

We conclude that approximately 4% of men with otherwise unexplained severe spermatogenic failure carry mutations in *NR5A1*. The data also suggest that some forms of male infertility may be an indicator of mild testicular dysgenesis, underlining a need for careful clinical investigation of men presenting with infertility and incongruous testosterone and gonadotropin levels.

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Web Resources

The URLs for data presented herein are as follows:

HGDP-CEPH Human Genome Diversity Cell Line Panel, http://www.cephb.fr/HGDP-CEPH-Panel
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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