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LRH-1 May Rescue SF-1 Deficiency for Steroidogenesis: An in vitro and in vivo Study

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LHR-1 · *NR5A1* mutations · SF-1 deficiency · Steroidogenesis

Abstract
Steroidogenic factor 1 (*NR5A1*/SF-1) mutations usually manifest in 46,XY individuals with variable degrees of disordered sex development and in 46,XX women with ovarian insufficiency. So far, there is no genotype-phenotype correlation. The broad spectrum of phenotype with *NR5A1* mutations may be due to a second hit in a gene with similar function to *NR5A1*/SF-1. Liver receptor homologue-1 (LRH-1/*NR5A2*) might be a good candidate. We performed in vitro studies for the interplay between SF-1, LRH-1 and DAX-1, expression profiles in human steroidogenic tissues, and *NR5A2* genetic studies in a cohort (11 patients, 8 relatives, 11 families) harboring heterozygote *NR5A1*/SF-1 mutations. LRH-1 isoforms transactivate the *CYP17A1* and *HSD3B2* promoters similarly to SF-1 and compensate for SF-1 deficiency. DAX-1 inhibits SF-1- and LRH-1-mediated transactivation. LRH-1 is found expressed in human adult and fetal adrenals and testes. However, no *NR5A2*/LRH-1 mutations were detected in 14 individuals with heterozygote *NR5A1*/SF-1 mutations. These findings demonstrate that in vitro LRH-1 can act like SF-1 and compensate for its deficiency. Expression of LRH-1 in fetal testis suggests a role in male gonadal development. However, as we found no *NR5A2*/LRH-1 mutations, the 'second genetic hit' in SF-1 patients explaining the broad phenotypic variability remains elusive.

Steroidogenic factor-1 (SF-1, encoded by the *NR5A1* gene) regulates adrenal and sex development and function [Schimmer and White, 2010]. *NR5A1* mutations manifest in patients with a very variable phenotype and are frequently detected in 46,XY individuals with disorders of sex development (DSD) and in 46,XX women with normal sexual development but primary ovarian insufficiency [Lourenço et al., 2009; Camats et al., 2012]. So far, no genotype-structure-function-phenotype correlation has been found for SF-1 variants. For *NR5A1*, identical mutations within families may lead to different phenotypes [Lourenço et al., 2009; Camats et al., 2012], and so-called severe mutations do not always cause a severe phenotype in patients [Camats et al., 2012]. Furthermore, although the DSD and ovarian insufficiency phenotypes are mostly caused by heterozygote mutations, no domi-

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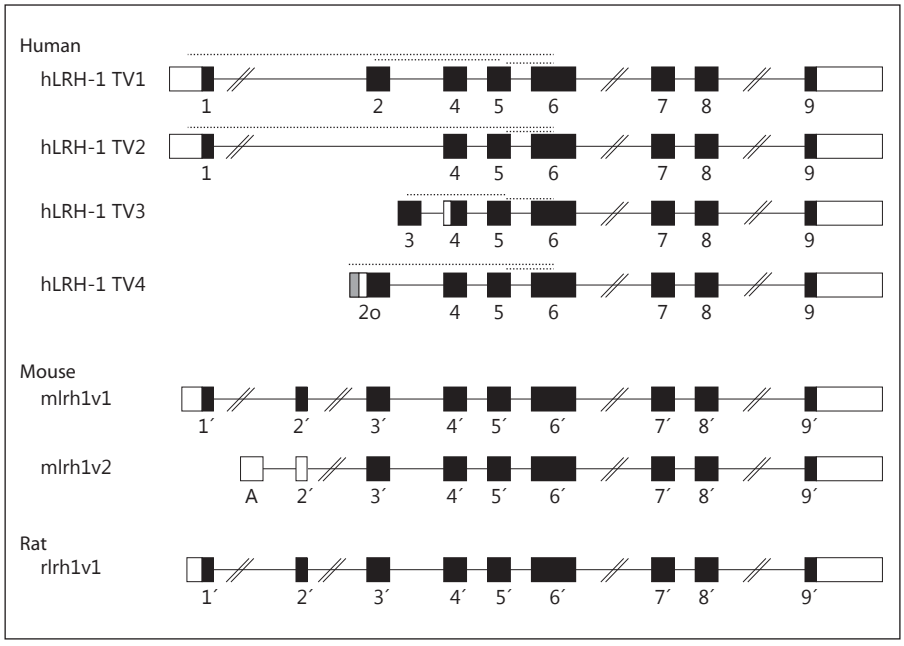

Fig. 1. Reported human and rodent LRH-1 transcripts. The schemes of 4 human and 3 rodent LRH-1 transcripts are shown (http://www.ncbi.nlm.nih.gov/) [Gao et al., 2006; Kawabe et al., 2013]. Dashed lines indicate the location of the PCR fragments amplified for the expression study.

nant negative effect has been detected [Camats et al., 2012]. Therefore, the broad phenotype of *NR5A1*/SF-1 mutations remains a conundrum.

The SF-1 interactome comprises a large number of transcriptional, postranslational and signaling (co-)modulators [Hoivik et al., 2010; Schimmer and White, 2010]. SF-1 interacts with numerous genes including developmental switches [Schimmer and White, 2010] and epigenetic factors [Hoivik et al., 2013]. Thus, the broad phenotype of *NR5A1* mutations may be due to a second hit in an interacting gene with similar function to SF-1. A double hit in both genes would then cause the severe phenotype, while a heterozygote *NR5A1* mutation alone would only cause a milder phenotype or even be compensated fully by the second gene when normal. We chose liver receptor homolog-1 (LRH-1, encoded by *NR5A2* gene) for our 'second hit' candidate for the following reasons: (1) both transcription factors LRH-1 and SF-1 belong to the same NR5A family of transcription factors [Fayard et al., 2004], (2) they have a high sequence similarity [Fayard et al., 2004], (3) both bind to the same promoter-binding cis-elements [Wang et al., 2001; Kim et al., 2004; Dubé et al., 2009], (4) they share common target steroidogenic genes [Wang et al., 2001; Sirianni et al., 2002; Peng et al., 2003; Fayard et al., 2004; Kim et al., 2004, 2005; Yazawa et al., 2009], and (5) both have a partially overlapping tissue expression pattern [Sirianni et al., 2002; Falender et al., 2003]. To date, no human mutations in *NR5A2* have been described and, to our knowledge, no DSD patients have been analyzed for *NR5A2* sequence variations.

LRH-1 and SF-1 share target genes related to steroidogenesis and sex development such as *STAR* [Sirianni et al., 2002; Kim et al., 2004; Dubé et al., 2009], *CYP11A1* [Kim et al., 2005], *CYP17A1* [Wang et al., 2001; Sirianni et al., 2002; Yazawa et al., 2009], *HSD3B2* [Peng et al., 2003; Dubé et al., 2009], *CYP19A1* [Dubé et al., 2009], *CYP11B1* [Sirianni et al., 2002], *CYP11B2* [Sirianni et al., 2002], and *WNT4* [Zhang et al., 2013]. In females, LRH-1 seems to play a major role in follicle maturation, ovulation and pregnancy [Duggavathi et al., 2008; Zhang et al., 2013], while its role in males is still poorly understood [Pezzi et al., 2004; Guo et al., 2007; Sierens et al., 2010]. SF-1 and LRH-1 are coexpressed in human non-steroidogenic tissues (liver, pancreas and pituitary) [Li et al., 1998; Wang et al., 2001; Sirianni et al., 2002; Falender et al., 2003], and steroidogenic tissues (adrenals, testes, ovaries, and placenta) [Wang et al., 2001; Sirianni et al., 2002; Falender et al., 2003; Peng et al., 2003; Yazawa et al., 2009; Zhang et al., 2013]. LRH-1 is less expressed in adrenals than SF-1 [Sirianni et al., 2002; Falender et al., 2003; Yazawa et al., 2009]. SF-1 is highly expressed in all 3 steroidogenic tissues (adrenals > testes > ovaries) [Yazawa et al., 2009]. The expression pattern of SF-1 and LRH-1 within the gonads is complex [Sirianni et al., 2002]. In the ovary, SF-1 is expressed mainly in follicular theca and interstitial

cells in rodents [Ikeda et al., 1993; Falender et al., 2003; Liu et al., 2003] and human [Peng et al., 2003; Sato et al., 2003]. In contrast, LRH-1 is expressed mainly in follicular granulosa cells and the corpus luteum in rodents [Liu et al., 2003] and human [Peng et al., 2003]. In the human adult testis, the expression of SF-1 and LRH-1 is also cell-type specific and partially overlapping: Leydig cells express both SF-1 and LRH-1, Sertoli cells express SF-1, and germ cells (spermatocytes/spermatids) express LRH-1 [Yazawa et al., 2009]. Whether LRH-1 is expressed in the human fetal testis has not yet been investigated.

LRH-1 was cloned and characterized from human liver tissues [Li et al., 1998]. Initially, 3 different isoforms were described which derive from alternative splicing in adult and fetal human liver [Li et al., 1998; Zhang et al., 2001; Fayard et al., 2004] (fig. 1). Isoforms 1 and 2 (isoform 2 lacks exon 2) are considered active [Li et al., 1998] and are expressed in the human liver [Li et al., 1998] and in the rat testis [Pezzi et al., 2004]. Lately, a new isoform 3 has been entered in the NCBI database, which has not been further investigated so far (fig. 1). This variant has an alternate exon (exon 3) instead of exons 1 and 2, has a downstream translation start site in exon 4 and is shorter than isoforms 1 and 2. Yet another human isoform confined to granulosa cells (gc-LRH-1-2c) was recently reported [Kawabe et al., 2013]. This isoform contains a larger exon 2 formed by usage of an alternative transcription start site [Kawabe et al., 2013]. Additional 3 isoforms were found in embryogenic stem cells of mice and rats [Gao et al., 2006].

DAX-1 is a global negative transcriptional regulator of steroidogenesis [Lalli and Sassone-Corsi, 2003]. The expression pattern of DAX-1 is restricted to tissues directly involved in sexual determination, steroidogenesis and reproductive function [Ikeda et al., 1996; Hanley et al., 2001; Lalli and Sassone-Corsi, 2003; Sato et al., 2003]. DAX-1 expression is coincidental to SF-1 tissue expression [Lalli and Sassone-Corsi, 2003] and, in part, also to LRH-1 tissue expression in granulosa cells [Kawabe et al., 2013] and in Leydig cells [Yazawa et al., 2009]. DAX-1 has a specific function in distinct cell populations in male and female gonads [Lalli and Sassone-Corsi, 2003]. Its activity seems essential for gonadal development in males, but dispensable in females [Lalli and Sassone-Corsi, 2003]. DAX-1 represses SF-1 [Hanley et al., 2001; Iyer and McCabe, 2004] and LRH-1 [Kim et al., 2004] transactivation activity via protein-protein interaction [Ito et al., 1997; Hanley et al., 2001; Suzuki et al., 2003]. At the same time, SF-1 stimulates transcription of DAX-1 [Hoyle et al., 2002], thereby inhibiting its own expression. Moreover, SF-1 and LRH-1 regulate LRH-1 expression via promoter activation [Kawabe et al., 2013]. Overall, a complex triangular relationship between DAX-1, SF-1 and LRH-1 seems to play an important role in steroidogenesis and sex development.

This study aimed to elucidate the underlying cause for the broad phenotype of human *NR5A1*/SF-1 mutations. LRH-1 was hypothesized to be a modulator of SF-1 being able to replace its function in case of deficiency. To address this hypothesis, the ability of LRH-1 to transactivate steroidogenic promoter reporters in vitro was assessed and compared to wild-type and mutant SF-1, including their interplay with DAX-1. LRH-1 expression profiles were assessed for human adult and fetal adrenal and testis tissues. Finally, genetic studies for *NR5A2*/LRH-1 mutations were performed in a cohort of 14 individuals harboring heterozygote *NR5A1*/SF-1 mutations and 5 non-carrier relatives.

Patients and Methods

Genetic Analyses

For the genetic studies, written informed consent was obtained from all individual participants/legal guardians included in the study after full explanation of the purpose and nature of all the procedures used. The study was approved by the respective ethical committees of the University Hospital Bern, Switzerland and the Vall d'Hebrón University Hospital in Barcelona, Spain.

The *NR5A2* gene was sequenced in a cohort of 11 families with heterozygote *NR5A1* mutations and 10 were previously described [Camats et al., 2012]. All exon sequences and their flanking intronic sequences of the *NR5A2* (LRH-1) gene were amplified by PCR, using specific primers (online suppl. table 1; see www.karger.com/doi/10.1159/000381575). PCR products from *NR5A2* were sequenced using the BigDyeTerminator v3.1 Cycle Sequencing Kit on an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). Primers used for PCR were also employed for sequencing (online suppl. table 1). Obtained sequences were analyzed against GenBank entry NC_000001.10 *(NR5A2)*. Hardy–Weinberg equilibrium was tested for SNPs presenting 3 alternate genotypes according to standard procedures using the χ^2 test. Linkage disequilibrium between individual SNPs was calculated as the correlation coefficient between pairs.

Plasmids and Cloning

Promoter luciferase reporter vectors -3.7kbCYP17A1_Δluc, -1050HSD3B2_pGL3 and empty control vectors (Δluc, pGL3) were available from previous work [Huang and Miller, 2000; Flück and Miller, 2004]. Wild-type (WT) SF-1/*NR5A1*_pcDNA3 and V20L-mutant SF-1/*NR5A1*_pcDNA3 mammalian expression vectors were also available from previous work [Camats et al., 2012]. LRH-1/*NR5A2*_pCMX vectors (in this study named isoform 1/TV1 and isoform 2/TV2) were kindly provided by Dr. Schoonjans (École Polytechnique Fédérale, Lausanne, Switzerland), and hu-

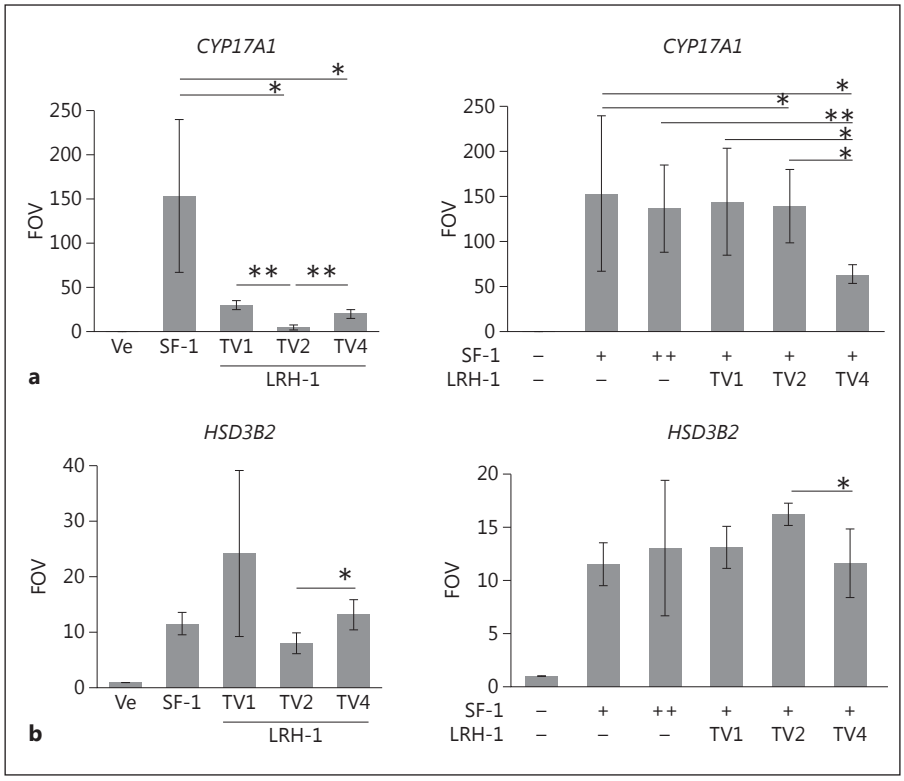

Fig. 2. LRH-1 can activate the steroidogenic *CYP17A1* and *HSD3B2* promoters. The ability of LRH-1 isoforms 1, 2 and 4 to activate steroidogenic enzyme promoter luciferase reporter constructs was tested in non-steroidogenic HEK293 cells and compared to WT SF-1. Cells were transiently transfected with *NR5A1*/SF-1 and *NR5A2*/LRH-1 expression vectors either alone or in combination, and together with a *CYP17A1* (**a**) or *HSD3B2* (**b**) promoter reporter construct. Luciferase activity was measured with the Promega Dual Luciferase assay system. Results are expressed as fold over empty vector (FOV), and represent the mean and SD of 2 to 5 independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$. LRH-1 isoforms are all WT.

man ovarian gc-LRH1 (in this study named isoform 4/TV4) vector was kindly provided by Dr. Yazawa (Faculty of Medical Sciences, Translational Research Center, University of Fukui, Fukui, Japan). The DAX-1 cDNA (NCBI NM_000475.4) was obtained by RT-PCR from RNA of human adrenal NCI-H295R cells (ATCC CRL-2128™) and cloned into the pcDNA3 mammalian expression vector (Invitrogen AG, Basel, Switzerland). Used primers are listed in online supplementary table 1.

In vitro Studies

Promoter activity experiments were performed in non-steroidogenic, human embryonic kidney cells HEK293 as previously described [Camats et al., 2012]. In brief, cells were cultured in DMEM, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% sodium pyruvate (Gibco, Paisley, UK). For transfection, cells were cultured on 24-well plates and transiently transfected with WT SF-1 and/or V20L SF-1, and/or LRH-1 isoforms 1, 2, or 4 and/or DAX-1 expression vectors together with *CYP17A1* or *HSD3B2* promoter luciferase reporter constructs using Lipofectamine 2000™ (Invitrogen). One µg of promoter luciferase vectors and 0.25 µg mammalian expression vectors were transfected per duplicate (4:1). In the experiments including 2 mammalian expression vectors, they were transfected in a proportion 1:1 (0.125 µg each). Forty-eight hours after transfection, cells were washed with PBS, lysed and assayed for luciferase activity with the Dual-Luciferase Reporter (DLR™) Assay System (Promega AG, Wallisellen, Switzerland) on a Veritas microplate Luminometer reader (Turner BioSystems Luminometer and Software by Promega). Specific *Firefly* luciferase readings were standardized against *Renilla* control readings. Experiments were repeated 2–5× in duplicates, and data were summarized giving the mean ± SD. Statistical significance was calculated using an unpaired t-test with Prism 4 (Graphpad software) and Microsoft Excel. Significance was defined as $p \leq 0.05$.

Gene Expression Studies

Adrenal and testicular tissue cDNA samples were all purchased from Amsbio (AMS Biotechnology (Europe) Limited, Abingdon, UK) and originated each from 4 healthy individuals. cDNA obtained from HepG2 cells (ATCC HB 8065™) served as control. Semiquantitative PCRs for LRH-1 (isoforms 1, 2, 3, and 4), SF-1, DAX-1, and GAPDH (35 cycles, n = 3) were carried out using the recommended concentrations of the purchased cDNAs and 50 ng for the HepG2 cDNA. Primers used are listed in online supplementary table 1, and the location of the corresponding PCR fragments are depicted in figure 1. PCR products were electrophoresed on an agarose gel and detected by ethidium bromide on a UV transilluminator (Alphaimager, Proteinsimple, Santa Clara, Calif., USA).

Results

Both LRH-1 and SF-1 Can Transactivate Steroidogenic Promoters

The ability of LRH-1 isoforms 1, 2 and 4 (TV1, 2, 4) to activate steroidogenic enzyme promoters was tested and

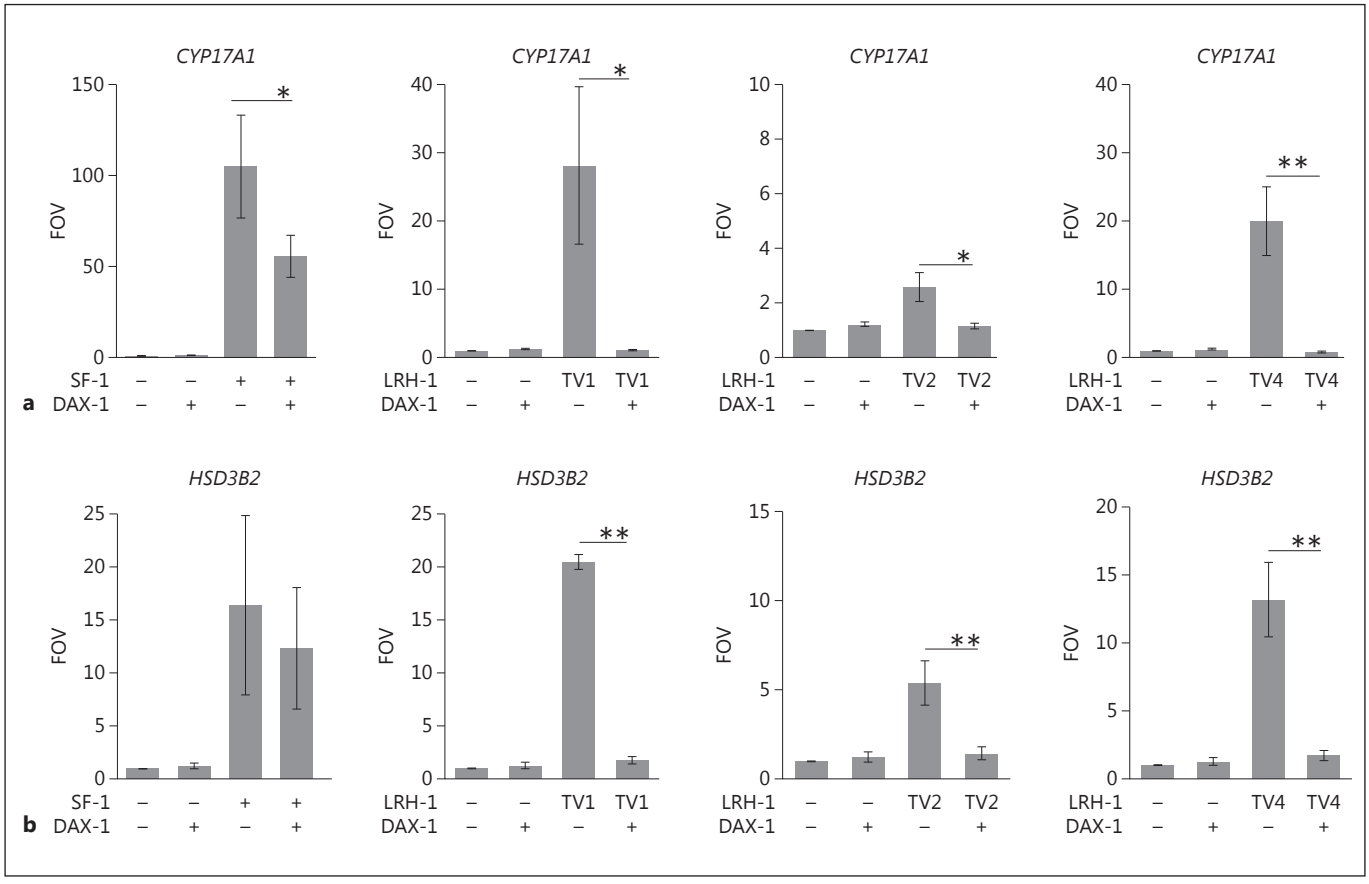

Fig. 3. The interplay between LRH-1, SF-1 and DAX-1 on promoter activities of steroidogenic genes. The ability of SF-1 and LRH-1 isoforms 1, 2 and 4 to activate steroidogenic enzyme promoter luciferase reporter constructs under the influence of DAX-1 was tested in non-steroidogenic HEK293 cells. Cells were transiently transfected with *NR5A1*/SF-1 or *NR5A2*/LRH-1 expression vectors with or without the DAX-1 expression vector and with the *CYP17A1* (**a**) or the *HSD3B2* (**b**) promoter reporter. Luciferase activity was measured with the Promega Dual Luciferase assay system. Results are expressed as described in figure 2. * $p < 0.05$, ** $p < 0.01$. LRH-1 isoforms are all WT.

compared to WT SF-1 (fig. 2). HEK cells were transiently transfected with *NR5A1*/SF-1 or *NR5A2*/LRH-1 (TV1, 2, 4) in combination with a *CYP17A1* (fig. 2a) or *HSD3B2* (fig. 2b) promoter reporter. These assays showed that all LRH-1 isoforms are able to transactivate both the *CYP17A1* and the *HSD3B2* promoters. Compared to SF-1, this transactivation seems to be weaker on the *CYP17A1* promoter than on the *HSD3B2* promoter, where similar results for LRH-1 and SF-1 were found. When transfecting cells with both SF-1 and LRH-1 mimicking homozygosity or double effect of heterozygosity for SF-1 and LRH-1, we found no additional effect, neither for double dosage SF-1 nor for the combination of SF-1 with any of the transcript variants of LRH-1 on both the *CYP17A1* (fig. 2a) and the *HSD3B2* (fig. 2b) promoters. Only LRH-1 TV4 seemed to impair the effect of SF-1 on *CYP17A1* promoter activity.

DAX-1 Inhibits Not Only SF-1 but Also LRH-1-Mediated Transactivation

DAX-1 is a well-known SF-1 modulator [Hanley et al., 2001; Iyer and McCabe, 2004]. Therefore, we wondered whether DAX-1 also modulates the effect of LRH-1 on transcription. HEK293 cells were transiently transfected with *NR5A2*/LRH-1 or *NR5A1*/SF-1 with or without DAX-1 and *CYP17A1* or *HSD3B2* promoter reporters (fig. 3). As expected, we found that DAX-1 impaired the effect of SF-1 on the *CYP17A1* promoter, less so on the *HSD3B2* promoter. Similarly, DAX-1 was able to inhibit the effect of all tested LRH-1 variants on both the *CYP17A1* and the *HSD3B2* promoters. In general, this inhibitory effect of DAX-1 was even stronger with LRH-1 partners than with SF-1.

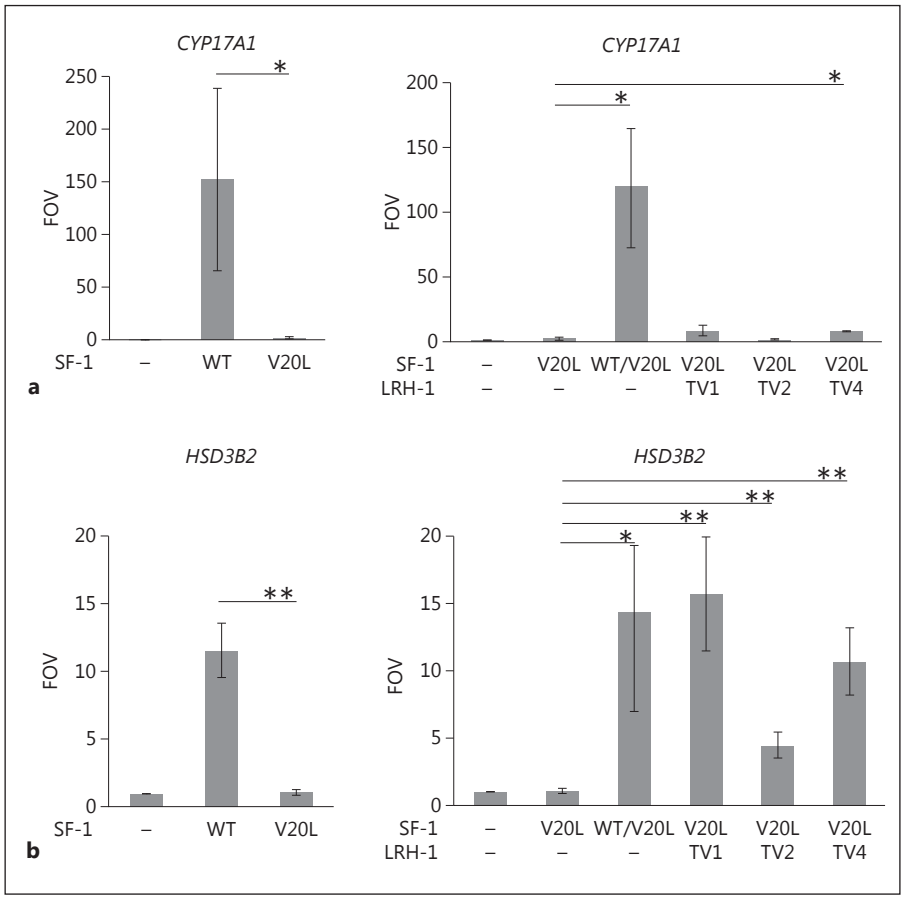

Fig. 4. LRH-1 can rescue SF-1 deficiency in vitro. Mutation V20L SF-1 loses almost all transcriptional activity on the *CYP17A1* and *HSD3B2* promoters [Camats et al., 2012]. Therefore, the ability of LRH-1 isoforms 1, 2 and 4 to rescue transactivation activity of V20L SF-1 on steroidogenic promoter reporters was tested in non-steroidogenic HEK293 cells and compared to WT SF-1. Cells were transiently transfected with *NR5A1*/SF-1 and *NR5A2*/LRH-1 expression vectors and *CYP17A1* (**a**) and *HSD3B2* (**b**) promoter reporter constructs. Luciferase activity was measured with the Promega Dual Luciferase assay system. Results are expressed as described in figure 2. * $p < 0.05$, ** $p < 0.01$. LRH-1 isoforms are all WT.

LRH-1 Can Rescue Mutant SF-1 in vitro

The V20L SF-1/*NR5A1* mutation was originally detected in heterozygote state in a family with a severely affected 46,XY DSD patient and a nonaffected carrier father representing the broad phenotype of *NR5A1* mutations. In previous studies, we showed that mutant V20L SF-1 loses almost all transactivation activity on the *CYP17A1* and *HSD3B2* promoters in HEK293 cells, and that cotransfection with WT SF-1 fully restores this activity [Camats et al., 2012]. Therefore, we were interested whether LRH-1 might also be able to rescue mutant SF-1 function. For that, HEK293 cells were again transfected with LRH-1 and the steroid enzyme promoter reporters, but this time with mutant V20L SF-1 (fig. 4). For *CYP17A1*, only LRH-1 isoform 4 was able to rescue some *CYP17A1* activity compared to WT SF-1 (fig. 4a). By contrast, for *HSD3B2*, we found that all LRH-1 isoforms were able to rescue the loss of function of mutant SF-1 similar to WT SF-1 (fig. 4b). The lowest effect was observed with LRH-1 isoform 2.

Gene Expression Pattern of SF-1 and LRH-1 in Human Steroidogenic Tissues

If LRH-1 and SF-1 collaborate for steroidogenesis, it is of interest how they are expressed in steroid producing tissues total. Therefore, we characterized the expression profile of LRH-1 and isoforms 1, 2, 3, and 4 in human adult and fetal adrenal and testicular tissues and compared it with the expression profiles of SF-1 and DAX-1. For this, we performed RT-PCRs using commercially available cDNAs of human tissues and HepG2 cDNA as control (fig. 5). A scheme of the 4 human and of the 3 rodent LRH-1 isoforms is shown in figure 1. LRH-1, SF-1 and DAX-1 were expressed in all investigated tissues (fig. 5). We describe the expression of LRH-1 in the human fetal testis for the first time. LRH-1 isoform 1 is expressed in all 4 tissues, lowest in the adult testis. Isoform 2 was difficult to assess and was finally only found in the fetal adrenal, but this might be a technical problem. Isoform 3 seems to be expressed at low levels in the adult adrenal and fetal testis. Interestingly, in contrast to other

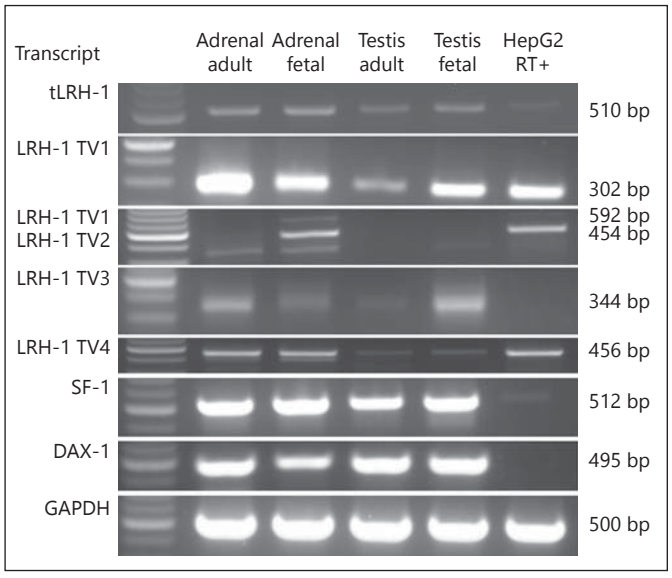

Fig. 5. Gene expression patterns of LRH-1 (isoforms 1–4), SF-1 and DAX-1 in human fetal and adult adrenal and testis tissues. Semiquantitative RT-PCRs were performed and a representative agarose gel picture is shown (n = 3). HepG2 served as positive experimental control. GAPDH was used as the internal control.

investigators [Kawabe et al., 2013], we found isoform 4 expressed in HepG2 cells. LRH-1 isoform 4, which is thought to be confined to the ovary [Kawabe et al., 2013], is also expressed in both adult and fetal adrenal tissues, and at low levels in adult and fetal testis.

No NR5A2 Mutations Were Detected in Patients with Heterozygote NR5A1 Mutations

We hypothesized that a second hit in the *NR5A2*/LRH-1 gene may be responsible for the broad phenotype seen in patients with heterozygote *NR5A1*/SF-1 mutations. Accordingly, the *NR5A2* gene was studied in our cohort of 11 families with heterozygote *NR5A1* mutations including 11 patients (ten 46,XY DSD patients and one 46,XX woman with ovarian insufficiency) and 8 relatives (3 carriers and 5 noncarriers) (table 1) [Camats et al., 2012]. Noncoding exon 3, which corresponds to isoform 3 with unknown function (http://www.ncbi.nlm.nih.gov), was not studied. Overall, genetic analysis of the *NR5A2* gene in our cohort did not reveal any mutations neither in the exons nor in their boundaries (table 1). We detected 11 sequence variants, 3 in exons and 8 in introns. These variants were considered SNPs as they were found in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp). In one individual, an additional intronic change in IVS1 (c.65–33C>T) was detected which was not included in the dbSNP (sister 3; table 1). The 3 exonic SNPs were silent changes (Lys111=; Pro250=; Asn523=). Three out of 8 intronic variants were located within the splicing-machinery region, and one of these (c.1230+14C>T, IVS7) was present as heterozygote and alternate homozygote in patients but also in their healthy relatives. Two patients presented the ancestral homozygote pattern for all the *NR5A1* sequence variants (patients 4 and 7); some patients had only few heterozygote changes, whereas others accumulated several alternate homozygote changes (patients 2, 5, 8, and 9).

NR5A2 genotype and allele frequencies were calculated for 11 affected *NR5A1*/SF-1 patients (table 1). The two IVS7 SNP genotypes were in linkage disequilibrium (82%). The SNPs were not considered relevant because they were found too frequently in the normal population, although 3 alternate homozygote genotypes (in IVS7 and IVS8) were more frequent in our population than in published data from several databases (table 1).

Discussion

Genotype-phenotype correlation for *NR5A1*/SF-1 mutations remains unsolved [Camats et al., 2012]. A second hit in another gene might explain the broad range of clinical manifestation. In our study, we therefore addressed the question whether the second hit is in the *NR5A2*/LRH-1 gene because LRH-1 is a close family member of SF-1 and has known regulatory functions in steroidogenesis [Sirianni et al., 2002; Dubé et al., 2009; Yazawa et al., 2009]. In our cell model, we found that LRH-1 has similar transactivational activity on steroid enzyme promoters as SF-1, that it interacts with DAX-1 leading to a repression of transactivation, and that both LRH-1 and SF-1 wild-types are able to compensate for V20L SF-1, a previously shown severe loss of function *NR5A1* mutation. In addition, we characterized the tissue expression of LRH-1 in fetal and adult adrenals and testes showing for the first time that the fetal testis expresses LRH-1 together with SF-1 and DAX-1. Nevertheless, we found no human mutations of *NR5A2*/LRH-1 in our cohort of heterozygote *NR5A1*/SF-1 patients or carriers.

Both LRH-1 and SF-1 are nuclear factors belonging to the NR5A family of transcription factors. Previous in vitro studies have shown that LRH-1 and SF-1 may regulate the same cis-elements in promoters [Peng et al., 2003] and that they can be differentially regulated themselves, thus competing for the same binding sites in target pro-

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Table 1. *NR5A2*/LRH-1 SNPs detected in *NR5A1*/SF-1 and relatives

Patients and relatives (n = 19)	*NR5A1*/SF1	rs113762480 IVS c.202+18G>T	rs41300849 IVS4 c.322–11C>T	rs34231860 Exon 5 c.333G>A p.Lys111=	rs2817008 IVS5 c.463+50A>G	rs2363454 IVS5 c.463+54G>T	rs2821368 Exon 6 c.750C>G p.Pro250=	rs3828110 IVS 7 c.1230+14C>T	rs10494808 IVS7 c.1231–64C>T	rs3762398 IVS8 c.1378+37G>A	rs1060060 Exon 9 c.1569C>T p.Asn523=
Patient 1	Val20Leu/WT	GG	CC	GG	AA	GG	*CG*	*CT*	*CT*	*GA*	**TT**
Mother1	WT/WT	GG	CC	GG	*AG*	GG	*CG*	CC	CC	GG	*CT*
Father 1	Val20Leu/WT	*GT*	*CT*	GG	*AG*	*GT*	CC	*CT*	*CT*	*GA*	*CT*
Patient 2	His24Tyr/WT	*GT*	CC	GG	AA	GG	CC	**TT**	**TT**	*GA*	*CT*
Patient 3	His24Thrfs*51/WT	GG	CC	GG	*AG*	GG	*CG*	**TT**	*CT*	*GA*	*CT*
Sister 3[a]	His24Thrfs*51/WT	*GT*	*CT*	GG	*AG*	*GT*	CC	CC	CC	GG	*CT*
Patient 4	Cys30Trp/WT	GG	CC	GG	AA	GG	CC	CC	CC	GG	CC
Patient 5	Gly90Arg/WT	GG	CC	GG	AA	GG	CC	**TT**	**TT**	**AA**	**TT**
Mother 5	Gly90Arg/WT	GG	CC	GG	AA	GG	CC	**TT**	**TT**	*GA*	*CT*
Patient 6	Pro130Argfs*165/WT	GG	CC	GG	AA	GG	*CG*	CC	CC	GG	CC
Father 6	WT/WT	GG	CC	GG	AA	GG	*CG*	CC	CC	GG	*CT*
Mother 6	WT/WT	GG	CC	GG	AA	GG	CC	*CT*	*CT*	*GA*	CC
Patient 7	Gln206Thrfs*20/WT	GG	CC	GG	AA	GG	CC	CC	CC	GG	CC
Patient 8	Leu231_233dup/WT	GG	CC	GG	AA	GG	CC	**TT**	**TT**	*GA*	*CT*
Father 8	WT/WT	GG	CC	*GA*	*AG*	GG	*CG*	*CT*	*CT*	*GA*	**TT**
Mother 8	WT/WT	GG	CC	GG	*AG*	GG	*CG*	*CT*	*CT*	GG	CC
Patient 9	Pro235Leu/WT	GG	CC	GG	AA	GG	CC	**TT**	**TT**	**AA**	*CT*
Patient 10	Trp302Stop/WT	GG	CC	GG	AA	GG	*CG*	*CT*	*CT*	*GA*	*CT*
Patient 11[b]	Arg313Cys/WT	*GT*	*CT*	GG	GG	*GT*	*CT*	*CT*	CC	GG	*CT*
Our patient population (n = not/a)		GG 81.8% GT 18.2% TT 0.0% G = 0.909 T = 0.091	CC 90.9% CT 9.1% TT 0.0% C = 0.9545 T = 0.0455	GG 100.0% GA 0.0% AA 0.0% G = 1.0 A = 0.0	AA 81.8% AG 9.1% GG 9.1% A = 0.8635 G = 0.1365	GG 90.9% GT 9.1% TT 0.0% G = 0.9545 T = 0.0455	CC 54.5% CG 45.5% GG 0.0% C = 0.7725 G = 0.2275	CC 27.3% CT 27.3% TT 45.4% C = 0.4095 T = 0.5905	CC 36.4% CT 27.2% TT 36.4% C = 0.50 T = 0.50	GG 36.4% GA 45.4% AA 18.2% G = 0.591 A = 0.409	CC 27.3% CT 54.5% TT 18.2% C = 0.5455 T = 0.4545
Population studies[c]		CSAgilent (N = 171) GG 87.2% GT 12.8% TT 0.0% G = 0.936 T = 0.064	CSAgilent (N = 1,227) CC 85.2% CT 14.0% TT 0.8% C = 0.922 T = 0.078	ESP (N = 4,524) GG 96.0% GA 3.8% AA 0.2% G = 0.979 A = 0.021	HapMap CEU (N = 226) AA 47.8% AG 37.2% GG 15.0% A = 0.664 G = 0.336	Pilot CEU (N = 120) G = 0.842 T = 0.158	ESP (N = 4,552) CC 50.5% CG 38.5% GG 11.0% C = 0.6975 G = 0.3025	HapMap CEU (N = 226) CC 22.1% CT 58.4% TT 19.5% C = 0.513 T = 0.487	HapMap CEU (N = 226) CC 22.1% CT 59.3% TT 18.6% C = 0.5175 T = 0.4825	HapMap CEU (N = 226) GG 53.1% GA 41.6% AA 5.3% G = 0.739 A = 0.261	CSAgilent (N = 1,306) CC(GG) 48.9% CT(GA) 43.4% TT(AA) 7.6% C = 0.706 T = 0.293

SNPs are numbered according to NM_205860.1 (NR5A2/LRH1 transcription variant 1). Heterozygotes are depicted in italics, and the alternate homozygotes in bold.

[a] Sister (46,XX) of patient 3 also had a change in IVS1 (g.200008753C>T; c.65–33C>T) not present in the dbSNP.

[b] Patient not included in Camats et al., 2012.

[c] Data source is the dbSNP from NCBI (www.ncbi.nml.nih.gov/snp).

CSAgilent = European-descent participants from the ClinSeqproject; ESP = cohort population from the Exome Sequencing Project; HapMap CEU = USA participants with Western and Northern European ancestry from the HapMap Project; N = chromosome count.

moters such as the inhibin α-subunit gene [Weck and Mayo, 2006]. Studies in stem cells demonstrated that LRH-1 and SF-1 act as key regulators of the steroidogenic lineage in human mesenchymal stem cells and that there is a similar time-dependent induction for LRH-1 and HSD3B2, but not for CYP17A1 [Yazawa et al., 2009].

Similar to our presented results, other investigators also found that SF-1-regulated genes are regulated by LRH-1. The effect of LRH-1 and SF-1 on steroid enzyme promoters *CYP17A1* and *HSD3B2* involved in androgen production was unambiguously stimulatory, although some differences in the strength of this effect were seen depending on the cell systems and experimental setup employed [Wang et al., 2001; Sirianni et al., 2002; Peng et al., 2003; Dubé et al., 2009; Yazawa et al., 2011]. We observed a weaker effect of LRH-1 on the *CYP17A1* promoter but a similar effect on the *HSD3B2* promoter when compared to SF-1 (fig. 2). We also observed that using longer promoter reporter constructs containing more putative LRH-1/SF-1 DNA-binding sites influenced the transactivation activity measured (data not shown). Therefore, only longer promoter constructs were used for our study similar to other studies which demonstrated that the *CYP17A1* promoter contains at least 3 regulatory cis-elements [Yazawa et al., 2009] and the *HSD3B2* at least 2 [Peng et al., 2003]. However, it has been shown that not all SF-1-binding elements are effective for LRH-1 or SF-1 transcriptional modulation, suggesting a more complex regulation [Kim et al., 2004; Weck and Mayo, 2006].

While the interplay of SF-1 with DAX-1 on transcription is well described [Hanley et al., 2001; Iyer and McCabe, 2004], less is known on this interplay between LRH-1 and DAX-1 [Peng et al., 2003]. Interestingly, we found that DAX-1 is a strong repressor of LRH-1-induced transactivation of the *CYP17A1* and a moderate repressor of the *HSD3B2* promoter (fig. 3). Differences on the effect of DAX-1 may be explained again by the number of effective DNA-binding sites in the regulated promoters [Hanley et al., 2001]. In addition, it has been shown that the binding affinity between the cofactors may modulate the transactivation effect, as stronger binding and effect was demonstrated between DAX-1 and SF-1 than for DAX-1 and LRH-1 [Suzuki et al., 2003].

LRH-1 and SF-1 can only collaborate in tissues where they have overlapping expression profiles. We studied the expression profile of the 4 reported LRH-1 isoforms in human fetal and adult adrenal and testis tissues and compared them to SF-1 and DAX-1 expression (fig. 5). Overall, LRH-1 is expressed in all human adult and fetal adrenals and testes with the highest expression for LRH-1 isoform 1 when compared to SF-1 and DAX-1. Isoform 1 was lowest in adult testis and isoforms 2, 3 and 4 were low expressed in all examined tissues. Control HepG2 cells expressed LRH-1 only, but no SF-1 and DAX-1. SF-1 is not expressed in adult rat liver [Falender et al., 2003], whereas DAX-1 has shown mixed results in mouse liver [Kawabe et al., 1999; Niakan and McCabe, 2005; Nedumaran et al., 2009]. The expression profile of LRH-1 and its isoforms for different organs, cells and species has been described in previous studies with variable results [Li et al., 1998; Wang et al., 2001; Sirianni et al., 2002; Peng et al., 2003; Fayard et al., 2004; Pezzi et al., 2004; Gao et al., 2006, 2007; Dubé et al., 2009; Yazawa et al., 2009; Sierens et al., 2010; Kawabe et al., 2013; Zhang et al., 2013]. This might be due to the fact that some isoforms are only expressed at very low levels, and for technical reasons, it is not easy to find a PCR strategy for the specific isoforms. In our hands, the most challenging was isoform 2. Previous studies showed that LRH-1 is expressed in human adult adrenals [Wang et al., 2001; Sirianni et al., 2002; Yazawa et al., 2009], fetal adrenals [Wang et al., 2001] as well as human adult testes [Sirianni et al., 2002; Yazawa et al., 2009] and ovaries [Peng et al., 2003; Yazawa et al., 2009; Zhang et al., 2013]. LRH-1 expression in human fetal testis is reported in our study for the first time; its expression in the developing testis has been only shown in rats so far [Guo et al., 2007; Sierens et al., 2010]. Human LRH-1 isoform 4 has only been reported recently [Kawabe et al., 2013]. It is described in ovarian tissue and cells as a granulosa cell-specific isoform (gc-LRH-1) which is not found in HepG2 liver cells [Kawabe et al., 2013]. We detected LRH-1 isoform 4 in adrenal, testis and liver (HepG2) demonstrating that it is not ovary specific. In granulosa cells, isoform 4 was also shown to be controlled by an alternate promoter which is located upstream of exon 2 and is under the control of both SF-1 and gc-LRH-1/isoform 4 [Kawabe et al., 2013]. Thus, alternate promoter usage may prompt variable expression of LRH-1 isoforms.

Human mutations for *NR5A2*/LRH-1 are not known. We searched for LRH-1 mutations in our cohort (11 families, 19 individuals) for subjects harboring heterozygote *NR5A1*/SF-1 mutations for explained reasons. We did not find disease-causing mutations, but some sequence variations which qualify for SNPs when comparing and analyzing with genetic data available for the normal population in several data bases (table 1). However, our genetic analysis which focused on exons and boundaries may miss (intronic) mutations.

Several studies have shown that LRH-1 plays an important role in female sexual and reproductive function [Zhang et al., 2013]. LRH-1 is differentially expressed in the female reproductive system [Falender et al., 2003; Zhang et al., 2013] and differentially expressed when compared to SF-1 [Falender et al., 2003; Fayard et al., 2004]. Heterozygote *NR5A1* mutations may cause ovarian insufficiency [Lourenço et al., 2009; Camats et al., 2012]. We thought that phenotypical variability in disease state may be explained by variable compensation through LRH-1. This hypothesis appears supported by our in vitro findings, but not by the genetic analysis of our patients. Similarly, LRH-1 seems to play a role in male sex development and function as it is expressed in fetal and adult testis and it regulates gene expression of *CYP17A1* and *HSD3B2,* which are essential for androgen production. Compared to SF-1, LRH-1 seems to have a partially overlapping expression profile in the testis regarding development [Pezzi et al., 2004; Guo et al., 2007; Yazawa et al., 2009; Sierens et al., 2010] and cell specificity [Pezzi et al., 2004; Sierens et al., 2010], but the exact interplay of both transcription factors is not known. Additionally, our study clearly shows that LRH-1 cannot compensate fully for heterozygote *NR5A1* mutations in humans, although this seems possible in vitro. Together with the finding that heterozygosity for mutant *NR5A1*/SF-1 in humans is able to cause disease while all tested in vitro systems cannot reflect this state, we still think that a second hit in yet another gene could explain the broad phenotype of *NR5A1*/SF-1 mutations. Therefore, in the era of next generation sequencing, we are now conducting exome sequencing analyses on several patients with identified *NR5A1*/SF-1 mutations in search for other candidate genes which may solve the conundrum of the broad phenotype in these DSD patients.

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