

Supplementary Appendix

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Supplement to: Achard C, Courtilot C, Lahuna O, et al. Normal spermatogenesis in a man with mutant luteinizing hormone. *N Engl J Med* 2009;361:1856-63.

SUPPLEMENTARY APPENDIX

SUPPLEMENTARY METHODS

DNA sequencing

DNA was extracted from the blood leukocytes of the proband and family members. The three exons of the gene encoding the beta subunit of luteinizing hormone (*LHB*) were amplified by polymerase chain reaction (PCR) using previously described primers (1) and automated sequencing was carried out as previously described (2).

Expression vectors

Sequences encoding the alpha subunit (α) and wild-type beta subunit of luteinizing hormone (LH β) were amplified from pM² α and pM²LH β vectors (3) using a PfuUltra high-fidelity DNA polymerase (Stratagene) and restriction sites for EcoRI or BglII were added by PCR-mediated mutagenesis at the 5' and 3' extremities, respectively. The fragments released by digestion with these two enzymes were inserted between the corresponding restriction sites of the pSG5 expression vector to obtain pSG5- α and pSG5-LH β . The nine bases deletion resulting in a deletion of three amino acids (Δ HPI) identified in the human *LHB* gene was introduced into pSG5-LH β , using the QuickChange II site-directed mutagenesis kit (Stratagene) to obtain pSG5-LH β Δ HPI. The same method was used in pSG5- α , to insert the V5 epitope at the C-terminus of the α subunit, yielding pSG5- α -V5. All inserts were sequenced on both strands.

Immunohistochemistry

After deparaffinization tissue sections were microwaved in pH 6 citrate buffer at maximum power for 10 minutes and incubated overnight at 4°C with the primary antibodies. Antibody binding was detected with the LSAB+ (streptavidin-biotin-peroxidase-aminoethylcarbazole) kit (DakoCytomation), as previously described (20).

Primary antibodies used are listed below: polyclonals anti-3 β HSD (3-Beta-hydroxysteroid-dehydrogenase) (dilution 1:3000), polyclonal anti-P450c17 α (P450c17 alpha hydroxylase), polyclonal anti-Anti-Müllerian Hormone (AMH) (dilution 1:4000); monoclonal anti-proacrosine (10 μ g/ml), and monoclonal anti-Histone H1 (Genetex, Inc, dilution 1:100).

Hormonal assays

Androstenedione concentration was determined by radioimmunoassay (Immunotech). The intra-assay and inter-assay coefficients of variation were $\leq 8.1\%$ and $\leq 11.9\%$, respectively. Estradiol concentration was determined by radioimmunoassay (DiaSorin). The intra-assay and inter-assay coefficients of variation were between 2.6% and 6.1%, and between 4.6% and 6.1%, respectively. Estrone concentration was determined by radioimmunoassay (Diagnostic System Laboratories). The intra-assay and inter-assay coefficients of variation were between 4.4% and 9.4%, and 6% and 11.1%, respectively. Inhibin B and AMH concentrations were determined by sandwich enzyme-linked immunosorbent assays (OBI-DSL and Diagnostic System Laboratories respectively). The intra-assay and inter-assay coefficients were between 4.2% and 13.5%, and between 10.2% and 21.5% (21.5% for concentrations of 52 pg/ml), respectively, for inhibin B. The intra-assay and inter-assay coefficients of variation for AMH were between 2.4% and 4.6%, and between 4.8% and 8%, respectively. The α subunit was measured by immunoradiometric assay (Immunotech A Beckman Coulter Company). The sensitivity of the assay was 0.02 IU/L. The intra-assay and inter-assay coefficients of variation were between 4.3% and 6.8% and between 2.7 % and 19% (19% for

concentration of 0.40 UI/L) respectively. Men over the age of 40 would be expected to have values below 0.7 IU/l.

Measurement of steroid levels by GC/MS

Steroid levels were determined by gas chromatography/mass spectrometry (GC/MS) (4). Briefly, steroids were extracted from samples by adding 10 volumes of methanol. We added 2 nanograms of deuterated internal standards — $^2\text{H}_5$ -testosterone (for testosterone), $^2\text{H}_5$ -17 β -estradiol (for 17 β -estradiol) and epietiocholanolone (for all other screened steroids) — to the extract for steroid quantification.

Samples were purified and fractionated by a solid-phase extraction (SPE) procedure with recycling. The unconjugated steroid fraction was eluted with 5 ml methanol/H₂O (90/10, v/v) from C18 silica minicolumns (500 mg, International Sorbent Technology). Steroids were then separated by HPLC, using a P1000XR quaternary pump and an AS 100 XR TSP autoinjector (ThermoFisher Scientific). HPLC was carried out with a Lichrosorb Diol column (25 cm x 4.6 mm, 5 μm) column at 30°C. Steroids were eluted at a flow rate of 1 ml per minute, in a 90/10 (v/v) mixture of hexane and mixture A. The composition of the latter was 85 parts hexane to 15 parts isopropanol (v/v). Steroids were collected with a fraction collector (2002 model, Gilson) over a period of 10 to 31 minutes. Steroids were derivatized with heptafluorobutyric anhydride in anhydrous acetone for 30 minutes at 20°C, dried under a stream of N₂ and resuspended in hexane for GC/MS analysis. Calibration samples and biological samples were injected into the apparatus with an AS 3000 autosampler and analyzed with a Focus-DSQ-II (ThermoFisher Scientific) apparatus. Injection was performed in the splitless mode at 250°C (1 minute of splitless time), with the temperature of the gas chromatograph initially maintained at 50°C for 1 minute and then ramped up from 50 to 350°C at a rate of 20°C/minute. The flow rate of the helium carrier gas was maintained at 1 ml/minute throughout the analysis. The transfer line and ionization chamber temperatures were 280°C and 220°C, respectively. Electron impact ionization was used for mass spectrometry, with an ionization energy

of 70eV. Samples were analyzed by single ion monitoring (SIM) and the identification of each steroid was supported by its gas chromatography (GC) retention time and its two diagnostic ions. Testis and plasma steroid levels were determined from a previously established calibration curve for each steroid, based on internal standard peak area ratios.

Cell culture and transfection

Human embryonic kidney cells (HEK-293 and HEK-293T) and African green monkey kidney cells (COS-7) cells were cultured, unless otherwise stated, in complete Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific), 10% fetal bovine serum, 2 mM L-glutamine and 2 mM pyruvate, at 37°C, in a humidified incubator, under an atmosphere containing 5% CO₂. Cells were transiently transfected with plasmids prepared with the EndoFree Plasmid Kit (Qiagen SA), using SuperFect transfection reagent (Qiagen SA) according to the manufacturer's protocol.

Study of wild-type and mutant Luteinizing Hormone (LH) levels in eukaryotic transfected cells

HEK-293T cells were transiently transfected with equivalent amounts of pSG5- α and pSG5-LH β or pSG5-LH β Δ HPI expression vectors. The transfected cells were incubated in complete medium for 16 h and were then cultured for 48 h in 5 ml of serum-free DMEM / Ham's F-12 (1:1) medium supplemented with 2 mM L-glutamine, 0.5 μ g per liter insulin and 5 mg per liter apotransferrin (Sigma-Aldrich). The medium was then collected, supplemented with HEPES, pH 7.4 (20 mM final concentration) and protease inhibitors (Roche Diagnostics), and centrifuged to remove cell debris. We used Centriprep Centrifugal Filter Units with YM-10 membranes (Amicon, Millipore Corporation) to concentrate supernatants containing wild-type LH hormone or mutant LH hormone by factors of about 20 and up to 400, respectively. Concentrated culture medium from mock-

transfected cells was used as control. Aliquots (0.5 ml) of crude medium were stored for LH immunoassays in non-concentrated medium. Media were stored overnight at 4°C or for longer periods at - 80°C, until analysis. In parallel, cell lysates were prepared to study intracellular levels of LH β subunits by Western blot analysis. The concentration of secreted wild-type LH hormone in culture media was determined by immunoradiometric assays, according to the manufacturer's instructions. The kits used were RIA-gnost hLH (CIS Bio International), LHsp-ELISA (Biosource Europe), Coat-A-Count LH IRMA (DPC), LH IRMA kit (Immunotech). None of these assays recognized the mutant LH hormone.

For studies of intracellular levels of wild-type and mutant LH β subunits, transfected HEK-293T cells were lysed by incubation in 0.5 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% Triton X-100, protease inhibitors, 0.1 mM phenylmethylsulfonyl fluoride) per dish for 30 minutes on ice and disrupted by passage through a 22-gauge needle. The lysate was centrifuged for 30 minutes (15,000 g) at 4°C, and the concentration of protein in the supernatant was determined with a micro-BCA kit (Pierce). Cell lysates (70 μ g of total protein per sample) and samples of concentrated conditioned media were diluted in 2x SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 1.4 M β -mercaptoethanol, 0.2% bromophenol blue). Samples were denatured by heating and subjected to SDS-PAGE in reducing conditions, in a 15% polyacrylamide gel. The gel was blotted onto a membrane and LH β and LH β Δ HPI subunits were detected with an anti-hCG β antibody (Abcam ab14301; dilution 1:1500) that cross-reacts with human LH β . The membrane was incubated with a secondary antibody coupled to horseradish peroxidase, and immune complexes were visualized with the ECL detection system (Amersham Pharmacia). Quantity One 1-D analysis software (Bio-Rad) was used for quantification.

Measurement of wild-type and mutant Luteinizing Hormone bioactivities

The bioactivity of the wild-type and mutant LH hormones secreted into the medium of transfected HEK-293T cells was determined by assessing cAMP accumulation after the addition of various amounts of this medium to HEK-293 cells transiently expressing the human Luteinizing Hormone receptor (LHR). We dispensed 5×10^5 HEK-293 cells per well into six-well dishes coated with poly(allylamine hydrochloride) (Sigma-Aldrich). The cells were transfected with the expression vector pSG5-LHR and cultured for 48 h in complete medium. The day of stimulation, cells were incubated for 2 h in incubation medium (DMEM, 20 mM Hepes pH 7.4, gelatin 1 mg per milliliter) at 37°C, and then for a further 30 minutes in 1 ml of the same medium supplemented with 0.5 mM isobutylmethylxanthine (IBMX). Cells were stimulated with concentrated culture media, prepared as described above and containing recombinant wild-type or mutant LH hormones. As only the wild-type LH hormone could be quantified with immunoassays, we first estimated the amount of mutant LH β subunits relative to the amount of wild-type LH β subunits in culture media by western blotting. Concentrated media containing similar amounts of wild-type or mutated LH β subunits were then added to cells expressing the human LH receptor. Various dilutions of concentrated culture medium from mock-transfected HEK-293T cells and stimulation medium alone were also used as negative controls. cAMP was quantified with a Biotrak assay system (GE Healthcare).

Coimmunoprecipitation experiments for studying wild-type and mutant LH β subunits dimerization with the α subunit

COS-7 cells were transiently transfected with equimolar amounts of pSG5- α -V5 and pSG5-LH β or pSG5-LH β Δ HPI expression vectors. The cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% Triton X-100, protease inhibitors, 0.1 mM phenylmethylsulfonyl fluoride) 48 hours later, and lysed on ice for 1 h with a Potter homogenizer. The cell lysate was centrifuged at 13,000g and 4°C for 15 minutes and precleared by incubation for

1 h with protein G beads (Amersham Pharmacia Biotech) at 4°C. Supernatants were collected, protein concentration was determined and equal amounts of protein were incubated overnight at 4°C with 5 µg per milliliter of anti-V5 antibody (Invitrogen) (5) or 5 µg per milliliter of a non immune immunoglobulin as a control. Complexes were collected by incubation with protein G beads. Proteins bound to the beads were eluted, denatured and subjected to SDS-PAGE in a 15% polyacrylamide gel. In parallel, equal amounts of proteins from non-immunoprecipitated cell lysates were subjected to SDS-PAGE, to compare levels between samples. Immunodetection was carried out with an anti-V5-HRP antibody (Invitrogen), a polyclonal anti-β-hCG antibody (Abcam ab14301), and an anti-β-actin antibody (Abcam), followed by a secondary antibody coupled to horseradish peroxidase (HRP-linked donkey anti-rabbit or sheep anti-mouse antibodies (Amersham Pharmacia); HRP-linked rabbit anti-chicken antibodies (Pierce)). Complexes were visualized with the ECL detection system (Amersham Pharmacia).

SUPPLEMENTARY RESULTS

FIGURE S1. Sequence and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis for the family presenting hypogonadism and *LHB* gene mutation

In Panel A, sequencing of the *LHB* gene of the proband (lower sequence, IV-3) and its comparison with the sequence of this gene from the control (upper sequence, Ctl) shows a nine-base deletion (arrow) resulting in the deletion of three amino acids from the protein. This deletion creates a BsrDI restriction site. Panel B shows the PCR-RFLP analysis confirming the results of the sequencing analysis. Amplicons obtained by PCR with the forward (F) and reverse (R) primers (see Fig. 1, panel B), containing the complete coding region of the *LHB* gene including exons E1 to E3 and introns I1 to I2, were digested with BsrDI, making it possible to discriminate between fragments found in wild-type *LHB* genes (1252 bp) and in *LHB* genes with deletions at the heterozygous

(1252, 654 and 589 bp) and homozygous (654 and 589 bp) states. Ctl: Control DNA. MW: molecular weight standard markers are indicated on the right, in base pairs (bp).

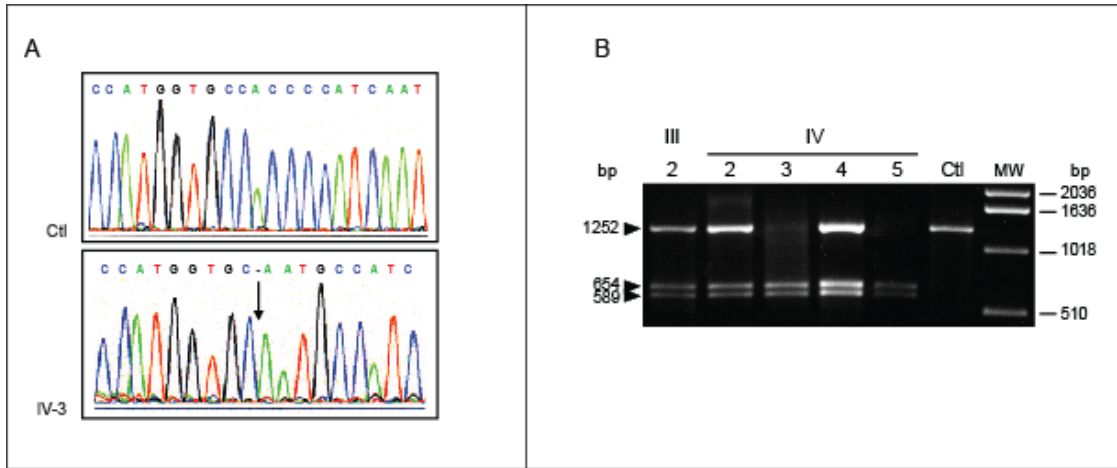


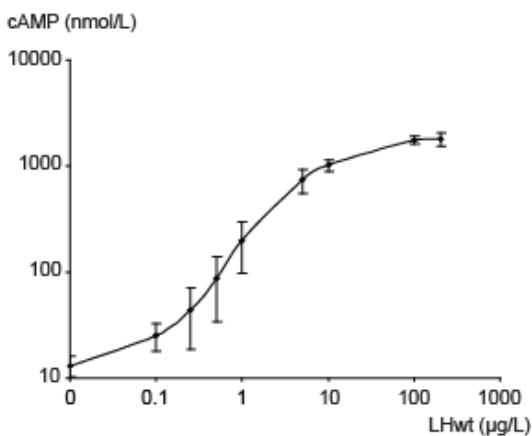
TABLE S1. Intratesticular hormones concentrations in the proband

Testosterone, 5 α -dihydrotestosterone, androstenedione, androstenediol and 17 β -estradiol concentrations were determined by gas chromatography/mass spectrometry (see details in Supplementary Methods). The intra-assay variation coefficient was between 5.0% and 10.0% and the inter-assay variation coefficient was between 7.5% and 11.0%, for all the steroids investigated. Control: age-matched control testes. The concentrations obtained for the control are consistent with previous reports (6).

Subject	Testosterone ng/g	5 α -dihydrotestosterone ng/g	Androstenedione ng/g	Androstenediol ng/g	17 β -estradiol ng/g
IV-3	20.2	0.59	1.01	1.59	0.11
Control	356.6	12.5	15.3	99.0	0.87

FIGURE S2. Dose-response curve generated after stimulation of HEK-293 cells expressing the Luteinizing Hormone receptor (LHR) with increasing concentrations of wild-type LH hormone

To compare the bioactivity of wild-type LH and mutant LH hormones produced in HEK-293T cells cotransfected with α and LH β subunit expression vectors, a standard dose-response curve was generated following the stimulation of HEK-293 cells expressing the human LH receptor with different concentrations culture medium containing wild-type LH hormone and quantification of cAMP accumulation. Wild-type LH was produced in HEK-293T cells cotransfected with α and LH β subunit expression vectors. The wild-type LH hormone concentration in culture medium was determined by immunofluorometric assay. The values shown are the means \pm SD of three independent experiments. The accumulation of cAMP was maximal for an LH concentration of 100 μ g per liter and the 50% maximal effective concentration (EC_{50}) was about 6 μ g per liter, as previously reported (1). Comparative quantification of wild-type LH β and mutant LH β (LH $\beta\Delta$ HPI) subunits secretion was carried out by western-blot analysis (see Fig. 3A).



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