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ORIGINAL RESEARCH

Follicle-stimulating hormone receptor (*FSHR*) alternative skipping of exon 2 or 3 affects ovarian response to FSH

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ABSTRACT: Genes critical for fertility are highly conserved in mammals. Interspecies DNA sequence variation, resulting in amino acid substitutions and post-transcriptional modifications, including alternative splicing, are a result of evolution and speciation. The mammalian follicle-stimulating hormone receptor (*FSHR*) gene encodes distinct species-specific forms by alternative splicing. Skipping of exon 2 of the human FSHR was reported in women of North American origin and correlated with low response to ovarian stimulation with exogenous follicle-stimulating hormone (FSH). To determine whether this variant correlated with low response in women of different genetic backgrounds, we performed a blinded retrospective observational study in a Turkish cohort. Ovarian response was determined as low, intermediate or high according to retrieved oocyte numbers after classifying patients in four age groups (<35, 35–37, 38–40, >40). Cumulus cells collected from 96 women undergoing IVF/ICSI following controlled ovarian hyperstimulation revealed four alternatively spliced FSHR products in seven patients (8%): exon 2 deletion in four patients; exon 3 and exons 2 + 3 deletion in one patient each, and a retention of an intron 1 fragment in one patient. In all others (92%) splicing was intact. Alternative skipping of exons 2, 3 or 2 + 3 were exclusive to low responders and was independent of the use of agonist or antagonist. Interestingly, skipping of exon 3 occurs naturally in the ovaries of domestic cats—a good comparative model for human fertility. We tested the signaling potential of human and cat variants after transfection in HEK293 cells and FSH stimulation. None of the splicing variants initiated cAMP signaling despite high FSH doses, unlike full-length proteins. These data substantiate the occurrence of FSHR exon skipping in a subgroup of low responders and suggest that species-specific regulation of FSHR splicing plays diverse roles in mammalian ovarian function.

Key words: FSHR / alternative splicing / cumulus cells / IVF or ICSI / ovarian response

Introduction

Follicle-stimulating hormone (FSH) is a key hormone of mammalian reproduction, necessary for gonadal development, maturation at puberty and for gamete production during the reproductive phase of life. FSH acts through the FSH receptor (FSHR) that is primarily expressed by cumulus and mural granulosa cells (Oxberry and Greenwald, 1982; Tisdall *et al.*, 1995; Liu *et al.*, 1998; Abel *et al.*, 2000; Saint-Dizier *et al.*, 2007). Inactivating mutations in humans and mice demonstrated that FSH signaling is essential for ovarian function and fertility but also for secondary sexual characteristics and body stature that depend on ovarian function (Matthews *et al.*, 1993; Aittomaki *et al.*, 1995; Kumar *et al.*, 1997; Layman *et al.*, 1997; Beau *et al.*, 1998; Dierich *et al.*, 1998; Touraine *et al.*, 1999; Abel *et al.*, 2000; Doherty *et al.*, 2002; Allen *et al.*, 2003; Meduri *et al.*, 2003).

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FSH is widely used in assisted reproductive technologies; however, the individual ovarian response to FSH varies significantly, ranging from recruitment of low to high numbers of follicles, even after following the same treatment regimen (Fauser *et al.*, 2008). Several biomarkers have been assessed as predictors of ovarian response to FSH, including basal serum FSH (during the early follicular phase), antral follicle count and serum anti-Mullerian hormone (AMH; Scott *et al.*, 1989; Bancsi *et al.*, 2003; Nelson *et al.*, 2007; Broer *et al.*, 2011, 2013).

Because of the central role of FSH signaling in ovarian stimulation, we and other investigators have previously hypothesized that alterations in the FSHR may contribute to the variability of ovarian response to FSH. The *FSHR* gene contains two single nucleotide polymorphisms (SNPs; c.919A/G and c.2038A/G; dbSNP: rs6165 and rs6166), resulting in two amino acid substitutions in the coding region (p.307Thr/Ala and p.680Asn/Ser) and a variation in the promoter of FSHR (g.-29G>A) (Simoni *et al.*, 1999; Wunsch *et al.*, 2005; Desai *et al.*, 2011). The association of these SNPs with ovarian homeostasis and IVF outcome has been extensively studied but their association with a phenotype is not consistent in all studies (reviewed in Mohiyiddeen and Nardo, 2010; Lalioti, 2011).

As an alternative to SNP-based studies of FSHR function, we have previously studied the FSHR mRNA isolated from cumulus cells (Gerasimova *et al.*, 2010). This approach has the advantage of detecting changes in regulatory, in addition to coding regions, that can result in post-transcriptional aberrations of FSHR expression, such as variations in alternative splicing. We identified four FSHR splicing variants in women undergoing infertility treatment with IVF (Gerasimova *et al.*, 2010). All variants were co-expressed with the normal receptor and none of the patients showed more than one variant. Interestingly, one of the splice variants (deletion of exon 2) was found only in patients with low response to FSH. These *FSHR* splice variants were unable to generate intracellular increases in cyclic adenosine monophosphate (cAMP) in response to FSH when expressed in mammalian cells *in vitro*, and co-expression of the del ex2 variant together with wild-type (WT) receptor resulted in lower intracellular cAMP compared with the WT alone (Gerasimova *et al.*, 2010; Lalioti *et al.*, 2010).

Deletion of exon 2 has not been detected in normal human tissue, while in several species *FSHR* transcripts are known to undergo alternative splicing affecting their extracellular domain encoded by exons 1–9 (Neubauer *et al.*, 2006) Interestingly, two isoforms of *FSHR* mRNA exist in the cat, a full-length and a shorter transcript missing exon 3 (Neubauer *et al.*, 2006) that contributes to the area involved in FSH binding (Heckert *et al.*, 1992; Fan and Hendrickson, 2005).

Environmental factors, such as seasonal variations, have been postulated to affect fertility in human and other mammals (Stolwijk *et al.*, 1994; Comizzoli *et al.*, 2003). Seasonality has been studied extensively in the cat, and it has been demonstrated that cumulus–oocyte complexes (COCs) recovered during seasonal anestrus require 10 times more FSH to achieve *in vitro* maturation (IVM) and developmental competence compared with those collected during the breeding season (Comizzoli *et al.*, 2003). The compromised developmental competence observed in cat oocytes matured *in vitro* during the non-breeding season also occurred in counterpart oocytes matured *in vivo* when cats were stimulated during the non-breeding season (Hobbs *et al.*, 2012). Seasonally mediated variation in FSHR expression (mRNA or protein) has been demonstrated in males of other species [black bear (Howell-Skalla *et al.*, 2000); sheep (Sanford *et al.*, 2002); bat (Hayashi *et al.*, 2002)] where reductions are directly correlated to a decline in spermatogenesis. Therefore, it seems that *FSHR* transcript alternative splicing may occur both naturally and as part of inadequate follicle performance in a clinical setting.

Because of the clinical significance of the variant lacking exon 2 found in low responders, we aimed to explore whether genetic and environmental factors influence the occurrence of this variant. Here, we report the analysis of FSHR mRNA in cumulus cells of patients treated in an IVF center in Ankara, Turkey, and thus of different genetic background and geographic location. Patients treated using both agonists and antagonists were included to test for pharmacologic influence. Using expression in mammalian cells *in vitro*, we explored the capability of FSHR encoded by a transcript lacking exon 2 (found in women with low response to FSH) or exon 3 (found in women with low response to FSH and also identified as a naturally occurring variant in the cat) to initiate signaling in response to a range of FSH doses.

Materials and Methods

Patient selection and cumulus cells collection

Patients were recruited from Gazi University School of Medicine IVF Center. The procedure was approved by the Gazi University Institutional Review Board committee (HIC protocol: 131/11.05.2011).

A total of 96 women undergoing infertility treatment with IVF and ICSI between June 2010 and October 2011 at the Gazi University School of Medicine, IVF unit (Ankara, Turkey) were included in this study. The causes of infertility were male factor, female factor (tubal factor, anovulation and endometriosis), unexplained infertility, or a combination of male and female factors.

In agonist cycles, treatment was initiated by pituitary suppression with GnRH agonists during the luteal phase of the preceding cycle. Stimulation with gonadotrophins was initiated only after down-regulation had been achieved (estradiol level <50 pg/ml in the absence of ovarian cysts on ultrasonography). In antagonist cycles, treatment was initiated on Day 3 of the cycle after ultrasonographic evaluation of the ovaries revealed the absence of ovarian cysts and serum progesterone levels were found to be <1 ng/ml. The stimulation protocol included 150–300 IU/day of gonadotrophins, either recombinant (Gonal F, Merck Serono, Turkey or Puregon, Merck Sharp Dohme, Turkey) or in combination with hMG (Menogon, Erkim, Turkey, or Merional, IBSA, Turkey). Patients received human chorionic gonadotrophin (hCG; Ovidrelle 250, Merck Serono, Turkey) when there were two or more follicles >18 mm in diameter with accompanying follicles >14 mm and an adequate E2 response. Oocytes were collected 36 h after hCG injection. All patients tested in this study were treated with ICSI.

Retrieved COCs were placed in dishes with culture medium (G-MOPS, VitroLife, Sweden) where cumulus cells were dissected mechanically using two G30 needles, one for holding the oocyte and the other for dissecting the cumulus cells surrounding the oocyte. The dissection process was carried out without hyaluronidase enzyme. The cumulus cells of each patient were pooled together into a single eppendorf tube. Each sample was washed twice with 0.5 ml phosphate-buffered saline (PBS), centrifuged at 1000*g* for 1 min and the supernatant was removed after each wash. After the last wash, the pellet was resuspended in 50 μl SideStep™ Lysis and Stabilization Buffer (Stratagene, USA). The samples were kept at −80°C until analysis.

RNA extraction and cDNA synthesis

Trizol (500 μl; Invitrogen, Carslbad, CA, USA) was added to the cumulus cells/Sidestep samples and they were lysed using a homogenizer. RNA

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was extracted according to the manufacturer's instructions. Briefly, 100 µl chloroform was added to each sample, mixed and incubated at room temperature for 3 min. The samples were then centrifuged at 12 000*g* for 15 min at 4°C. The upper phase, containing the RNA, was precipitated with 250 µl isopropanol after centrifugation at 12 000*g* for 10 min at 4°C. The pellet was washed with 500 µl 70% ethanol (in DEPC-water), centrifuged at 8000*g* for 10 min at 4°C and air-dried. The RNA was dissolved in 30 µl of DEPC-water and stored at −80°C until used.

To obtain cDNA, 5 µl of RNA (containing ∼200–500 ng of RNA) was mixed with 2 µl (20 pmol) random hexamers (Integrated DNA Technologies, Coralville, Iowa, USA) and 8 µl DEPC-water. The mix was denatured at 65°C for 5 min and chilled on ice, before the addition of the remaining reagents according to the manufacturer's instructions (Omniscript, Qiagen, Gaithersburg, MD, USA). The reaction was incubated at 37°C for 1 h.

Polymerase chain reaction

As an internal control, the expression of beta-actin (genomic sequence NG_007992 and mRNA sequence NM_001101) was detected by PCR using primers Act5F (exon 5) : 5′ GAC CTC TAT GCC AAC ACA GT 3′ and Act3R (exon 6) : 5′ TTG CTG ATC CAC ATC TGC T 3′. Exons 1–4 of the FSHR were amplified using nested PCR. Primary PCR was performed with primers FSHR.1F: 5′ GGA GGT TTT TCT CTG CAA ATG CAG 3′ and FSHR.11R: 5′ CAG ATA TTG AAG GTT GGG AAG 3′ and secondary PCR was performed with primers FSHR.19F: 5′ ATG GCC CTG CTC CTG GTC TC 3′ and 11R (as above). Amplifications were carried out using 25–35 cycles of PCR, with the following touch-down program: initial denaturation 94°C/5 min; 10 cycles of 92°C/30 s, 65°C/20 s (−1°C per cycle) and 72°C/30 s; 15–25 cycles of 92°C/30 s, 55°C/20 s and 72°C/30 s, in a reaction mix containing 1 µl cDNA, 1× PCR buffer (Qiagen), 125 µM dNTPs, 0.5 µM primers and 1 unit of Taq polymerase (Qiagen, Gaithersburg, MD, USA). Positive and negative controls were included in every RT–PCR reaction. The negative control contained all PCR materials except for template. The positive control included cDNA made from the human pre-luteal cell line KGN, which is known to express FSHR (Nishi *et al.*, 2001). KGN cells were kindly provided by Drs Nishi and Yanase (Fukuoka University, Japan). All PCR products were separated on 3% agarose/TAE gels and visualized using ethidium bromide staining.

Gel extraction, sequencing and analysis

The full-length and spliced forms of FSHR RT–PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol, eluted in 50 µl of water, and prepared for sequencing using a thermal cycling protocol with primer FSHR.19F, fluorescently labeled dideoxynucleotides (Big Dye Terminators) and Taq FS DNA polymerase (ABI). Sequence analysis was carried out on Applied Biosystems 3730 capillary instruments at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Sequencing results were compared with human FSHR transcript sequences using Sequencher software version 4.8 (Gene Codes, Ann Arbor, MI, USA) and Gene Construction Kit 3.5 (Textco BioSoftware Inc., West Lebanon, NH, USA).

Cell culture and mammalian expression constructs

Human KGN cells were maintained in MEMα with GlutaMax (Invitrogen) containing 5% heat-inactivated fetal bovine serum and antibiotic–antimycotic (Invitrogen). The cells were grown at 37°C with 5% CO_2 and passaged 4-fold once per week. RNA extraction and cDNA and synthesis were as described above for patient samples.

Human Embryonic Kidney cells (HEK293) were maintained in DMEM containing 4500 mg/l glucose, 110 mg/l pyruvate, 548 mg/l L-glutamine, 5% fetal bovine serum and antibiotic–antimycotic (Invitrogen). The cells were grown at 37°C with 5% CO_2 and passaged 10-fold once per week.

Construction of the human expression constructs is described in Gerasimova *et al.* (2010). A hemagglutinin (HA) tag was fused in-frame at the 3′ end of all FSHR variants both human and cat. The cat FSHR expression constructs were designed *in silico* using the Gene Construction Kit 3.5 (Textco biosoftware Inc.) and cat sequences with accession numbers NCBI: AY521181 and AY524543. The full-length cDNAs of the cat WT ($cFSHR^{WT}$) and truncated FSHR ($cFSHR^{ex3del}$) were inserted into vector pcDNA3.1 + hygro. They were synthesized by GeneArt plasmid services (Life technologies corporation; Invitrogen).

Transient transfections and cAMP measurements

To measure cAMP increase in response to stimulation, the cells were transiently transfected with pHTS-CRE (Biomyx Technology), which carries the firefly luciferase gene under the control of four cAMP response elements (CRE). They were co-transfected with the pRL-CMV plasmid (Promega), which drives the expression of Renilla luciferase under the control of a constitutive CMV promoter to normalize for transfection efficiency.

HEK293 cells were seeded in 48-well plates the day before transfection and transiently transfected at 60–70% confluency with 100 ng pHTS-cre, 50 ng FSHR plasmid and 10 ng pRL-CMV per well using calcium phosphate precipitation according to Gerasimova *et al.* (2010). Briefly, plasmids (prepared with QIAGEN midi columns) were mixed with 1.5 µl $CaCl_2$ 1 M and water to a final volume of 8 µl. The DNA/$CaCl_2$ mixture was added dropwise to 8 µl of 2× Hepes-buffered saline [1.6% NaCl (wt/vol), 1.2% HEPES (wt/vol), 0.04% Na2HPO4 (wt/vol), pH 7.12]. Eighteen to 24 h after transfection, the medium was replaced with 0.25 ml of UltraCHO (BioWhittaker, Inc., Walkersville, MD) supplemented with antibiotic–antimycotic (Invitrogen). Eight hours later, the cells were stimulated with recombinant human FSH (0–100 mIU/ml; follitropin beta, GonalF, Serono), forskolin (10 µM; Fisher Scientific), porcine pituitary FSH (0.02–2 U/ml; Sigma Aldrich; Cat# F2293) or, highly purified ovine FSH [0.5–5 µg/ml; NIDDK (National Institute of Diabetes & Digestive & Kidney Disease), Torrance, CA, USA] in UltraCHO media for 16–18 h. Forskolin was used as a positive control, since it causes a receptor-independent intracellular cAMP increase. Mock transfections included 100 ng pHTS-cre, and 10 ng pRL-CMV per well and lacked any FSHR plasmid. The cells were lysed and luciferase activity of the two different genes was measured using Dual-Glo luciferase kit (Promega) on a 20/20 Luminometer (Promega). The measurement of the firefly luciferase was normalized to the Renilla luciferase to produce relative luciferase units, according to the manufacturer instructions. All results presented are the means of three independent experiments, each performed in triplicate. Statistical analysis was performed using Excel (Microsoft). The significance of differences between treatments was assessed using Student *t*-test. *P*-value of <0.05 was considered significant for the comparisons.

Western blotting

For western blotting, the cells were transfected with the FSHR expression constructs in 10 cm dishes as described above. The proteins were expressed for 48 h. The cells were washed in cold PBS and lysed at 4°C in lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 1 mM Na3VO4, 50 mM NaF and 1 tablet of protease inhibitor mixture (Roche Molecular Diagnostics; per 50 ml of buffer)]. Lysates were cleared by centrifugation at 12 000*g* for 30 min at 4°C. Protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA). Then, 10 µg of total protein was mixed with 2× sodium dodecyl sulfate (SDS) sample buffer [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 0.1%

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bromophenol blue, 10% mercaptoethanol], fractionated on a 8% SDS–PAGE gel and transferred to PVDF membrane (Bio-Rad Laboratories) at 30 V overnight. The membrane was blocked with 5% non-fat dry milk in TBS-T (Tris-buffered Saline with Tween-20; 10 mM Tris–HCl, pH 7.4 and 150 mM NaCl with 0.11% Tween-20) for 1 h at room temperature and incubated with primary mouse anti-HA antibody (1:1000; Upstate Biotechnology, Lake Placid, NY) overnight at 4°C, followed by secondary anti-mouse-horseradish peroxidase (HRP) antibody (1:10,000; Pierce, Rockford, IL) for 1 h at room temperature. All antibodies were diluted in 1% milk in TBS-T. The signals were detected using SuperSignal ECL (Pierce) and exposed to film (Kodak). The experiments were repeated at least three times. Western blots for beta-Actin (1:5000; 13E5; HRP-conjugated; Cell Signaling Technology) were used as controls

Statistical analysis

Statistical analyses were performed using Matlab (R2011b; The MathWorks, Natick, MA) considering four age groups of patients: <35, 35–37, 38–40 and >40. The percentiles of number of retrieved oocytes were calculated for each age group separately. Patients' response levels were evaluated accordingly where <25%, 25–75% and >75% percentiles were defined as poor, intermediate and high responders, respectively. The significance of the differences between Day 3 serum FSH levels, administered total FSH and serum estradiol level at hCG administration was assessed using one way analysis of variance among age groups and among responder groups within the same age group. Fisher exact test was used to compare the occurrence of $FSHR^{ex2del}$ in low, normal and high responders. Alpha error of <0.05 was considered significant for the comparisons.

Results

Classification of patient ovarian response

Individual ovarian response to FSH stimulation of women undergoing IVF shows significant variability, ranging from poor to high response and depends on age (Fauser *et al.*, 2008). Patients were recruited from an IVF center in Ankara, Turkey and were separated into four age groups: <35, 35–37, 38–40 and >40, consistent with the grouping used for outcome analysis by the Society for Assisted Reproductive Technologies (SART). Data were collected and compared only from patients treated with the protocol described in the Materials and Methods section. The number of oocytes per cycle defining the lower 25th and higher 75th percentile was determined for each age group (Fig. 1). Consequently, less than eight oocytes were classified as low response in the youngest group, while for women above 38 years this threshold was four oocytes. As expected, the mean number of oocytes retrieved per cycle declined with age. Low responders had higher FSH at the baseline (Supplementary data, Table SI). AMH was not measured. Consistent with the number of oocytes retrieved, high responders had higher serum estradiol levels at the day of hCG administration (Supplementary data, Table SII).

Next, we assessed the relationship between the ovarian response and the total amount of FSH used for controlled ovarian stimulation (Supplementary data, Table SIII). We observed a trend toward a higher amount of total FSH used in women with low response, although this was not statistically significant. This is likely to be a reflection of the IVF procedure, where the FSH dose of poor responders is raised to improve stimulation and the dose of high responders is lowered to avoid hyperstimulation. These data exclude the possibility of a linear relationship between the ovarian response and the FSH amount received. Therefore, in this patient population, we defined as low responders those patients that produced fewer oocytes than the 25% of their respective age group and as high responders those patients that produced more oocytes than the 75% of their group. The exact oocyte cut off numbers for each age group is shown in Fig. 1B.

FSHR splicing variants

We have previously found a variant of FSHR lacking exon 2 ($FSHR^{ex2del}$) in patients with low response to FSH. Because of the clinical significance of the finding, we recruited patients of a different genetic background from Ankara, Turkey to test whether FSHR alternative splicing was also occurring in them.

Cumulus cell collection was as shown in Fig. 2A and described in Materials and Methods. RNA was extracted from cumulus cells of 96 patients undergoing IVF/ICSI. RT–PCR for β-actin was used to assess the quantity and quality of isolated RNA. Eighty-seven percentage of the samples (84 of 96) were positive for actin and were further analyzed for *FSHR* exons 1–4. Amplification of the FSHR cDNA was carried out using nested PCR (Fig. 2B). The receptor was successfully amplified in 83 cases (86% of total patients) and visualized by agarose gel electrophoresis (Fig. 2B). Age and response of patients positive for FSHR RT–PCR are shown in Table I. When a second band was present in addition to the expected band, the PCR products were sequenced. PCR amplifications and sequencing were all performed blindly, by investigators who did not know the ovarian response status of the patients analyzed.

Sequencing was performed either by extracting and purifying the two bands from the agarose gel or by sequencing the total PCR product (Fig. 3A and B). Alternatively spliced products were identified in 8% of the patients (7/83) (Table I and Fig. 3). These included exon skipping of exons 2 (patients ANK-50, ANK-55, ANK-95, ANK-128), 3 (patient ANK-63), 2 + 3 (patient ANK-83) and insertion of a 66 bp fragment of intron 2 (patient ANK-141; Figs 2B and 3). All affected patients also expressed the full-length FSHR product and none of the patients contained more than one variant (Fig. 2B). Because the number of nucleotides on exons 2 and 3 is a multiple of 3, exon skipping is not expected to cause a frame-shift.

Correlation of FSHR variants with ovarian response to FSH

Clinical characteristics of patients who carried FSHR splicing variants are shown in Table II. Consistent with our previous investigations in patients from North America, Del ex 2 was only found in four of 26 patients with poor response (ANK-50, 55, 95 and 128); none of the patients with normal or high response ($n = 57$) showed Del ex 2 ($P < 0.05$).

Exon 3 deletion was found in one patient (ANK-64) with poor response. A combination of exons 2 and 3 deletion was found in one poor responder (ANK-83). None of the patients with normal or high response showed Del ex 3 or Del ex 2 + 3.

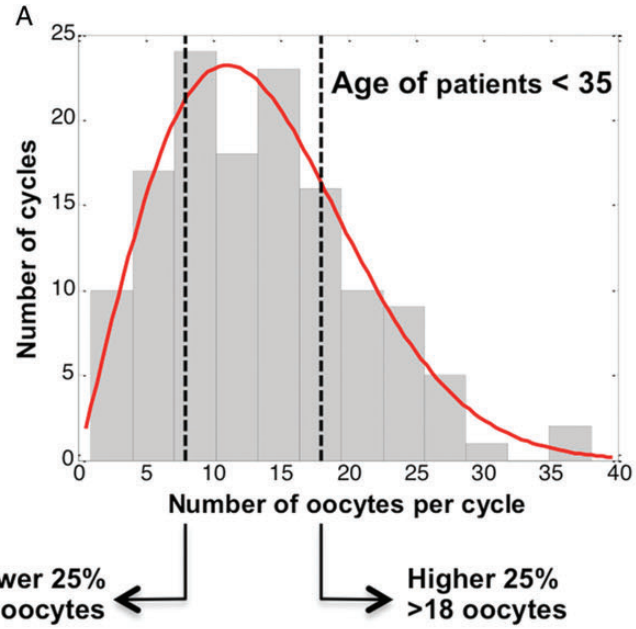
Finally, patient ANK-141 expressed a variant of FSHR with an insertion of 66 bp from intron 2 (IVS 2) between exons 2 and 3. Translation of this fragment would insert a premature stop codon immediately following exon 2 and would result in a non-functional protein.

Response of human FSHR del ex2 to FSH *in vitro*

Elevation of intracellular cAMP and stimulation of the cAMP-dependent protein kinase pathway is one of the main signaling pathways downstream of FSHR [reviewed in (Wood and Strauss, 2002)]. We have previously cloned the WT and del ex2 FSHR variants ($hFSHR^{WT}$ and

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A



B

Age group	Number of cycles	Mean ± SEM	Ovarian response (Quartiles of number of oocytes per cycle)		
			Poor responder (< 25%)	Intermediate responder (25% - 75%)	High responder (> 75%)
<35	135	13.8 ± 0.6	37 cycles <8 oocytes	63 cycles 8-18 oocytes	35 cycles >18 oocytes
35-37	24	11.1 ± 1.2	7 cycles <6 oocytes	11 cycles 6-16 oocytes	6 cycles >16 oocytes
38-40	19	8.9 ± 1.4	5 cycles <4 oocytes	9 cycles 4-12 oocytes	5 cycles >12 oocytes
>40	11	7.6 ± 1.7	2 cycles <3 oocytes	6 cycles 3-13 oocytes	3 cycles >13 oocytes

Figure 1 Number of oocytes retrieved per cycle following ovarian stimulation with FSH. (**A**) Graph of oocytes retrieved per cycle from patients under 35 years of age. The dotted lines represent the lower and higher 25th percentiles of the number of oocytes per cycle. (**B**) Classification of ovarian response and numbers of oocytes defining each category. Patients were classified into four age groups according to the society for assisted reproductive technologies (SART). SEM: standard error of the mean.

hFSHR^{ex2del}) in mammalian expression vectors and shown that the hFSHR^{ex2del} results in markedly decreased cAMP activation compared with the WT (Gerasimova *et al.*, 2010).

In order to assess whether the hFSHR^{ex2del} splice variant responds to higher doses of FSH, we tested the response *in vitro*. Western blotting showed that transient transfection in HEK293 cells resulted in expected

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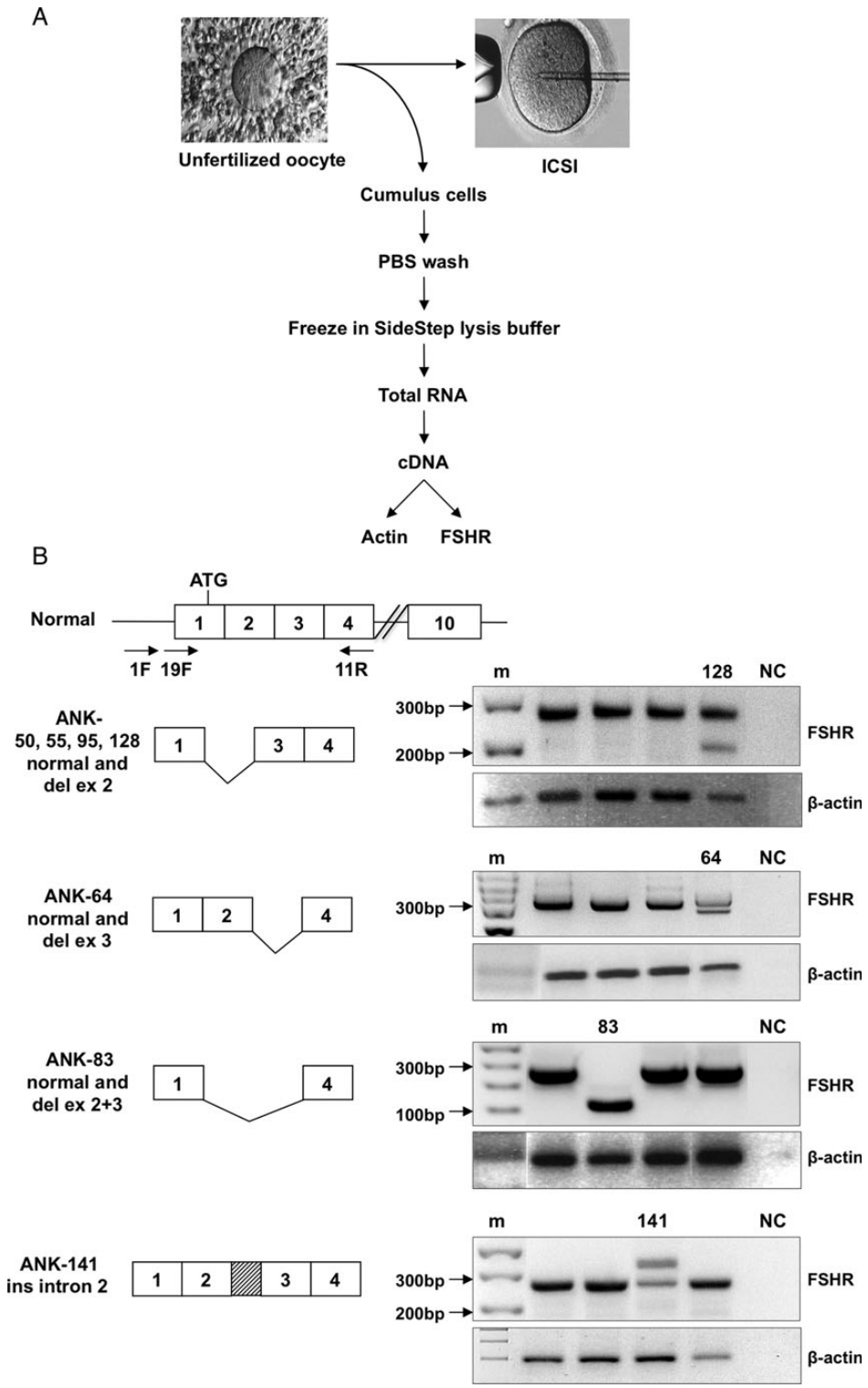


Figure 2 Cell isolation procedure and RT–PCR results. (**A**) Cumulus cells were mechanically isolated by dissection from retrieved oocytes prior to ICSI, washed in PBS and frozen in SideStep lysis solution until RNA isolation and RT–PCR. (**B**) Schematic representation of the PCR design and the location of primers on FSHR cDNA are shown on the top. Primary PCR was performed with primers 1F-11R and nested PCR with primers 19F-11R. Boxes represent exons and are numbered. For each splicing variant identified, a representative gel photo is shown. The RT–PCR for β-actin for the same samples is shown below the FSHR photo. Amplified products from four patients studied simultaneously are shown in each picture: three normal and one with an additional band. Patient ANK-83 showed only one shorter band the first time tested (B) and two bands when re-amplified (data not shown). It is likely that the single band found was due to preferential amplification of the shorter PCR product. m: DNA size marker; NC: negative control.

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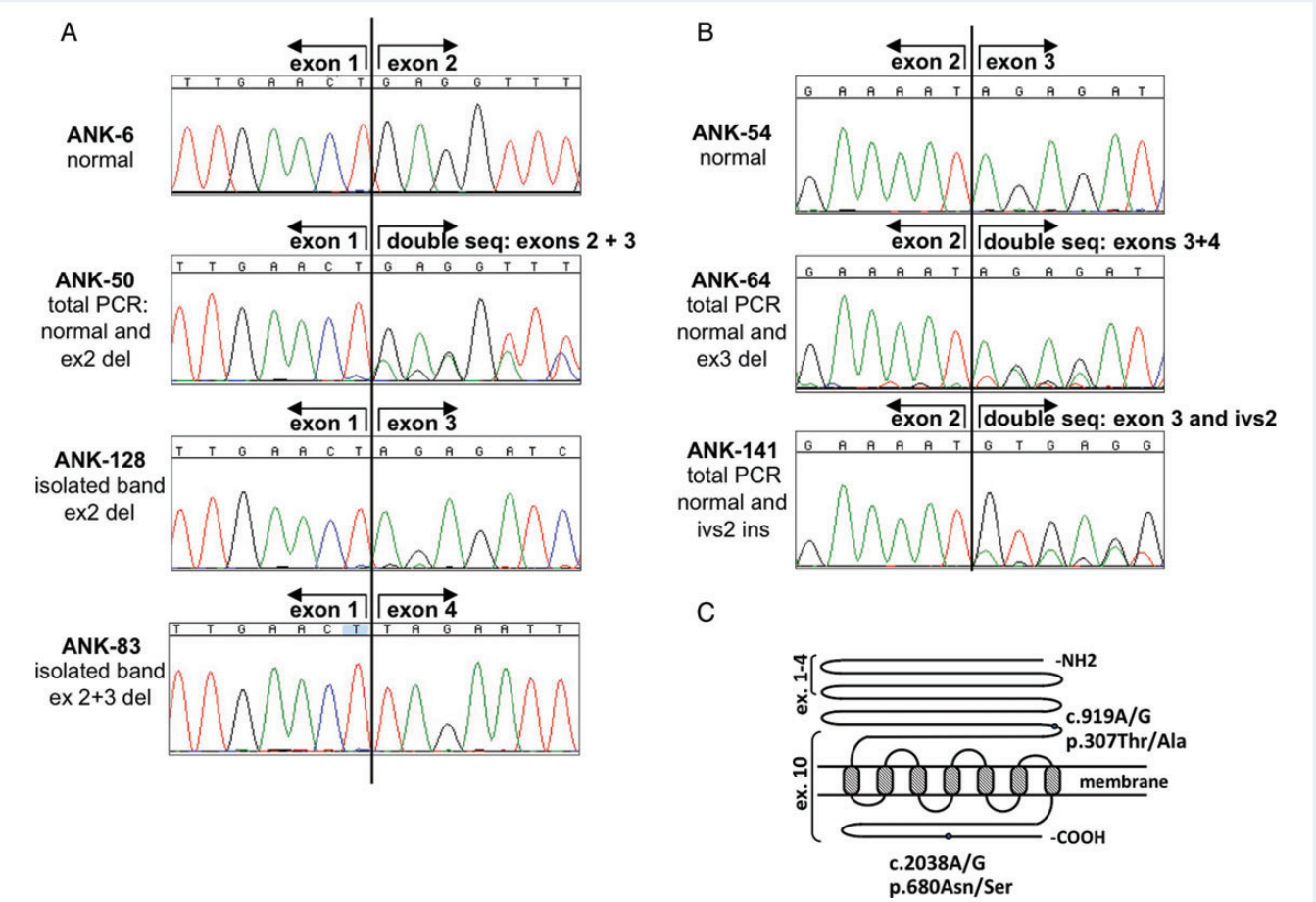


Figure 3 FSHR cDNA sequencing. Sequences of the bands or total PCR products shown in Fig. 2B. The type of product sequenced is indicated on the left of the chromatographs. The upper panels [from patient ANK-6 (A) or ANK-54 (B)] show the normal sequence. The limits of exons are indicated. (**A**) The left half of all sequences aligned is exon 1 while the second half varies according to the variant found. (**B**) Left half is exon 2 and second half varies. (**C**) Schematic representation of FSHR. The positions of the two common polymorphisms are indicated by black circles. c: cDNA, p: protein. The NH2-terminus constitutes the hormone-binding domain and is extracellular. The COOH-terminus is intracellular. Because of the genomic organization of the gene exons 1–9 encode most of the extracellular portion of the receptor and exon 10 encodes the remainder of the protein.

protein size considering the deletion of exon 2 (Fig. 4A). The band of the WT protein is broad because of glycosylation (Davis *et al.*, 1995; Guan *et al.*, 2010).

The transfected cells were stimulated with increasing amounts of FSH, including physiological and 10-fold higher doses (10–100 mIU/ml), and the amount of cAMP produced was quantified (Fig. 4B). The WT protein showed a dose–response to increasing amounts of FSH used to stimulate the cells. In contrast, the hFSHR^{ex2del} variant was unable to up-regulate cAMP production upon FSH stimulation, independent of the dose used. In order to verify that expression of the hFSHR^{ex2del} construct did not impair the ability of the cells to increase cAMP, we treated transfected cells with forskolin, a non-receptor mediated cAMP stimulant and showed a 10-fold elevation of cAMP production [data not shown; (Gerasimova *et al.*, 2010)].

Response of cat FSHR del ex3 to FSH *in vitro*

Interestingly, the del ex3 variant found in one patient is a common variant found in granulosa cells of the cat (Hobbs *et al.*, 2012). The sequence of human and cat FSHR proteins are extremely similar, with 91% identical amino acids and 95% conserved substitutions (Supplementary data, Fig. S1). Skipping of exon 3 does not change the reading frame but results in deletion of 25 amino acids from the extracellular region of the protein (Supplementary data, Fig. S1).

In order to assess the functional capacity of the cat splicing variant, we cloned the WT and truncated forms in the mammalian expression vector pcDNA3 (cFSHR^{WT} and cFSHR^{ex3del}; see Materials and Methods) and performed transient transfections in HEK293 cells. Expression of both forms was confirmed with western blotting (Fig. 5A).

We tested whether the cFSHR^{WT} protein could respond to human FSH, since cat FSH is not available. As expected by the high homology of the two receptors, the cat FSHR stimulated adequate cAMP production when treated with human FSH (Fig. 5B), although the response of the human receptor was more pronounced. In contrast, the deleted form of the cat receptor cFSHR^{ex3del} was unresponsive to any concentration of hormone (Fig. 5C). To exclude any subtle species variations, ovine and porcine hormones, which are commercially available, were also used with the same results (Fig. 5D).

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Discussion

Several aspects of mammalian reproduction are conserved among species. Therefore, genes critical for fertility, including the gonadotrophins and *FSHR*, share a high degree of sequence homology (Supplementary data, Fig. S1). Evolution and speciation account for the genetic differences observed between species. Sequence variation may convey a plethora of consequences including amino acid substitutions, expression level changes, post-transcriptional and post-translational modifications, including splicing and glycosylation. Besides genetic differences, epigenetic changes (environment, seasons and pharmacologic agents) can influence the function of the reproductive system in mammals including humans. In this study, we examined alternative forms of FSHR mRNA *in vivo* and *in vitro*. In particular, we confirmed that an alternatively spliced product of FSHR lacking exon 2, that we had previously found in 2 out of 10 low responders of North American origin (Gerasimova *et al.*, 2010), was also present in 4 out of 26 (15%) low responders of a different genetic background (Turkish) and treated at a different geographic location. Importantly, $FSHR^{ex2del}$ was absent from normal and high responders of the same genetic origin (0 out of 57). Moreover, while the low responders of North American origin (Gerasimova *et al.*, 2010) had all been treated with a GnRH agonist regimen, most of the Turkish patients with del ex2 were treated with a GnRH antagonist (Table II). Therefore, the exon skipping found in these patients is not likely to be caused by epigenetic factors (e.g. geographic location, pharmacologic), since the method of stimulation does not seem capable of modulating splicing. Our collective data provide robust evidence to implicate FSHR alternative splicing in regulation of ovarian response to FSH in a subgroup of low responders, independent of genetic background, local environment and type of ovarian stimulation.

In addition to exon 2 skipping found in several patients, exon 3 skipping was found in one low responder. While it is not possible to conclude whether this type of splicing is responsible for a phenotype, it is intriguing that exon 3 skipping occurs naturally in the cat (Hobbs *et al.*, 2012). In order to explore the role of these splice variants, we have cloned and expressed the human and cat WT genes and their respective splice variants *in vitro* and showed that they are unable to stimulate the main FSH signaling cascade, even using high doses of the hormones.

Post-transcriptional modification of mRNAs by alternative splicing is one mechanism by which transcriptomic complexity is generated from the surprisingly low number of genes currently estimated from the human genome sequence (Kalsotra and Cooper, 2011). As a result, tissue- and/or cell-specific isoforms, alternative intracellular localization, and thus altered biological function of proteins are observed. The splicing of the *CYP19A1* gene, encoding the aromatase enzyme that regulates the production of estrogen, is a well-studied example of alternative use of the first exon that dictates the tissue-specific expression (Bulun *et al.*, 2009). Recent studies based on RNA sequencing (RNA-seq) have shown that up to 90% of human genes undergo alternative splicing (Wang and

Table I Age and ovarian response of patients positive for FSHR RT–PCR.

	Number (%)
Age	
<35	55 (66%)
35–37	12 (14%)
38–40	9 (11%)
>40	7 (9%)
Total	83 (100%)
Ovarian response	
Low	26
Intermediate	39
High	18
PCR results	
Normal	76/83 (92%)
Abnormal	7/83 (8%)

Table II Clinical characteristics of patients carrying FSHR exon 1–4 splicing variants.

	Reason for IVF	Age	Oocytes	Day 3 FSH (IU/ml)	Day 3 E2 (pg/ml)	FSH received (IU)	E2 at stimulation (pg/ml)	Days of stimulation	Protocol	Pregnancy outcome
Del ex 2										
ANK-50	DOR	30	4	10.8	36	3500	1580	7	Antagonist[a]	+
ANK-55	DOR	32	2	9.5	35	4000	1100	11	Antagonist[a]	+
ANK-95	MALE	38	3	7.4	36	4500	1300	13	Antagonist[a]	–
ANK-128	UNEXP	32	5	4.9	75	2325	1033	10	Agonist	+
Del ex 3										
ANK-64	MALE	30	4	7.8	35	3000	1089	10	Antagonist[a]	+
Del ex 2+3										
ANK-83	AMA	47	3	11.0	42	4000	1380	9	Antagonist[a]	–
Ins IVS 2										
ANK-141	TUBAL	41	5	9.1	30	3950	1049	12	Agonist[a]	–

DOR, diminished ovarian reserve; UNEXP, unexplained; AMA, advanced maternal age.
[a]ANK-50; ANK-95; ANK-83 used Gonal-F. ANK-55; ANK-64; ANK-141 used Puregon.

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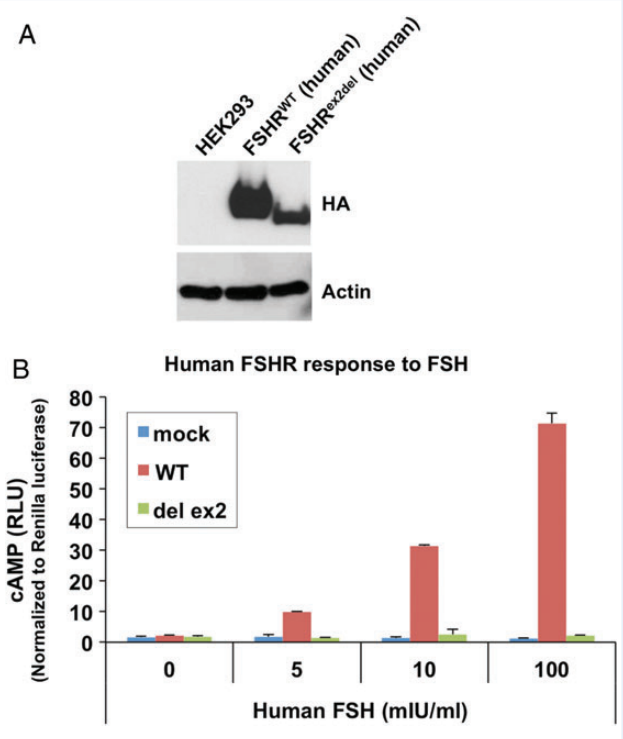


Figure 4 *In vitro* expression of human FSHR^{ex2del} and response to FSH. (**A**) Western blot analysis of transient transfections with human FSHR plasmids. Lane 1: Mock transfection of HEK293 cells. Lanes 2 and 3 HEK293 transfections with plasmids expressing human FSHR^{WT} and FSHR^{ex2del}. The receptor was tagged with hemagglutinin (HA) in the carboxyl-terminus. Top panel shows hybridization with an anti-HA antibody. Bottom panel shows a blot with the same lysates hybridized with an anti-β-actin antibody. (**B**) Luciferase increase in response to cAMP upregulation. In addition to FSHR plasmids, HEK293 cells were co-transfected with a cAMP-responsive luciferase plasmid (pHTS-CRE) and with a control plasmid (pRL-CMV) to normalize for transfection efficiency. Mock HEK293 cells were only transfected with pHTS-CRE and pRL-CMV. Unlike the WT protein, which showed a dose–response to FSH, the FSHR^{ex2del} protein was unable to elicit a response, even in doses of 100 mIU/ml.

Cooper, 2007; Nilsen and Graveley, 2010). Moreover, 15–60% of confirmed human mutations disrupt splicing (Wang and Cooper, 2007; Nilsen and Graveley, 2010). Sequence variation can account for intraspecies alternative splicing. We have previously demonstrated that a single nucleotide substitution (SNP) in the mouse *EPAB* mRNA can lead to the formation of an exonic splicing enhancer (ESE) and preferential splicing in two mouse subspecies (Seli *et al.*, 2008).

It is known that *FSHR* undergoes multiple splicing in most species that have been studied, including the human and the cat (Kelton *et al.*, 1992; Neubauer *et al.*, 2006). In spite of high sequence homology, there is extensive interspecies variation. For example, skipping of exons 2 and 5 has been observed in the mouse, and exons 4, 5 and 9 in cattle (O'Shaughnessy *et al.*, 1994; Rajapaksha *et al.*, 1996; Yaron *et al.*, 1998; Tena-Sempere *et al.*, 1999). In sheep and rat alternative splicing of the *FSHR* results in modification of the intracellular carboxyl terminus or production of a truncated soluble protein, which functions as a dominant negative receptor (Sairam *et al.*, 1996; Yarney *et al.*, 1997; Kraaij *et al.*, 1998). Despite the fact that both cattle and sheep are primarily mono-ovulatory species as is the human, there is some splicing variability between them. In human, although a syntenic region to the sheep exists in the genome, we were unable to detect a splicing product similar to the one found in sheep (Thanasoula and Lalioti, unpublished data). The cat *FSHR* shows alternative splicing of exon 3, one of the variants also found in patients, but its temporal expression does not correlate with the seasonal changes in fertility observed in cat (Hobbs *et al.*, 2012). Here, we showed *in vitro* that the cFSHR^{ex3del} variant was not active when expressed in HEK293 cells. The exact role of these isoforms is unknown, although we could postulate that they are involved in regulation of activity of the protein, by interacting with the normal full-length product (Sairam *et al.*, 1996).

In human, only skipping of exon 9 has been found in normal tissue [testis (Gromoll *et al.*, 1992)], while skipping of exons 6, 9 and insertion of intron 8 has been described in infertile males (Song *et al.*, 2002). In females, exon 2 skipping was found exclusively in a subgroup of low responders undergoing IVF [this paper and (Gerasimova *et al.*, 2010)]. In the current study, in addition to exon 2 skipping detected in four patients, we also detected combined exon 2 and 3 skipping in one patient, exon 3 skipping in one patient and retention of a 66 bp of intron 2 in one patient. The latter insertion is predicted to generate a premature stop codon and protein truncation. Since the number of nucleotides on exons 2 and 3 is a multiple of 3, exon skipping is not expected to cause a frame-shift. All of these variants are predicted to affect the extracellular, hormone-binding part of the receptor (Fig. 3C; Fan and Hendrickson, 2005; Huhtaniemi and Themmen, 2005). Sequencing did not identify the genomic defect that could cause exon skipping in our patients. It is possible that a mutation within the introns surrounding the affected exons could cause abnormal splicing as has been previously shown in other diseases (Faa *et al.*, 2009). However, introns 1 and 2 of the *FSHR* are very large (>50 kb) and were not sequenced. An alternative possibility is that aberrant splicing is caused by a mutation in a gene that encodes a splicing factor. We believe that this is less likely, because splicing factors mutations have more profound or multiple effects (Gonzalez-Santos *et al.*, 2008; Graubert *et al.*, 2012). Moreover, using polymorphic markers within the coding region of the gene in patients treated in several cycles, we have previously shown that exon skipping was not random and always affected the same allele of the patient (Gerasimova *et al.*, 2010), suggesting the presence of a *cis*-acting element. It is noteworthy that exon skipping is unlikely to be caused by the hyperstimulation protocol, because all patients tested (low or normal responders) were treated with the same protocol and mRNAs were analyzed in parallel.

The endogenous amount of FSHR protein expressed in the ovary is very small, and does not allow detection by western blot. *In vitro*, the FSHR^{ex2del} variant is adequately expressed and the protein is stable. Illegitimate and/or incomplete processing, including splicing, can affect the processing of the wild-protein when the two are expressed in the same cell. It has been previously shown that splice variants of the LH receptor interfere with trafficking of the full-length LHR and its expression on the cell surface and consequently with its ability to bind the hormone (Apaja *et al.*, 2006). FSHR is known to form dimers and we have shown that the FSHR^{ex2del} is unable to initiate a signaling cascade in response to FSH when expressed alone in mammalian cells (Urizar *et al.*, 2005; Gerasimova *et al.*, 2010; Guan *et al.*, 2010).

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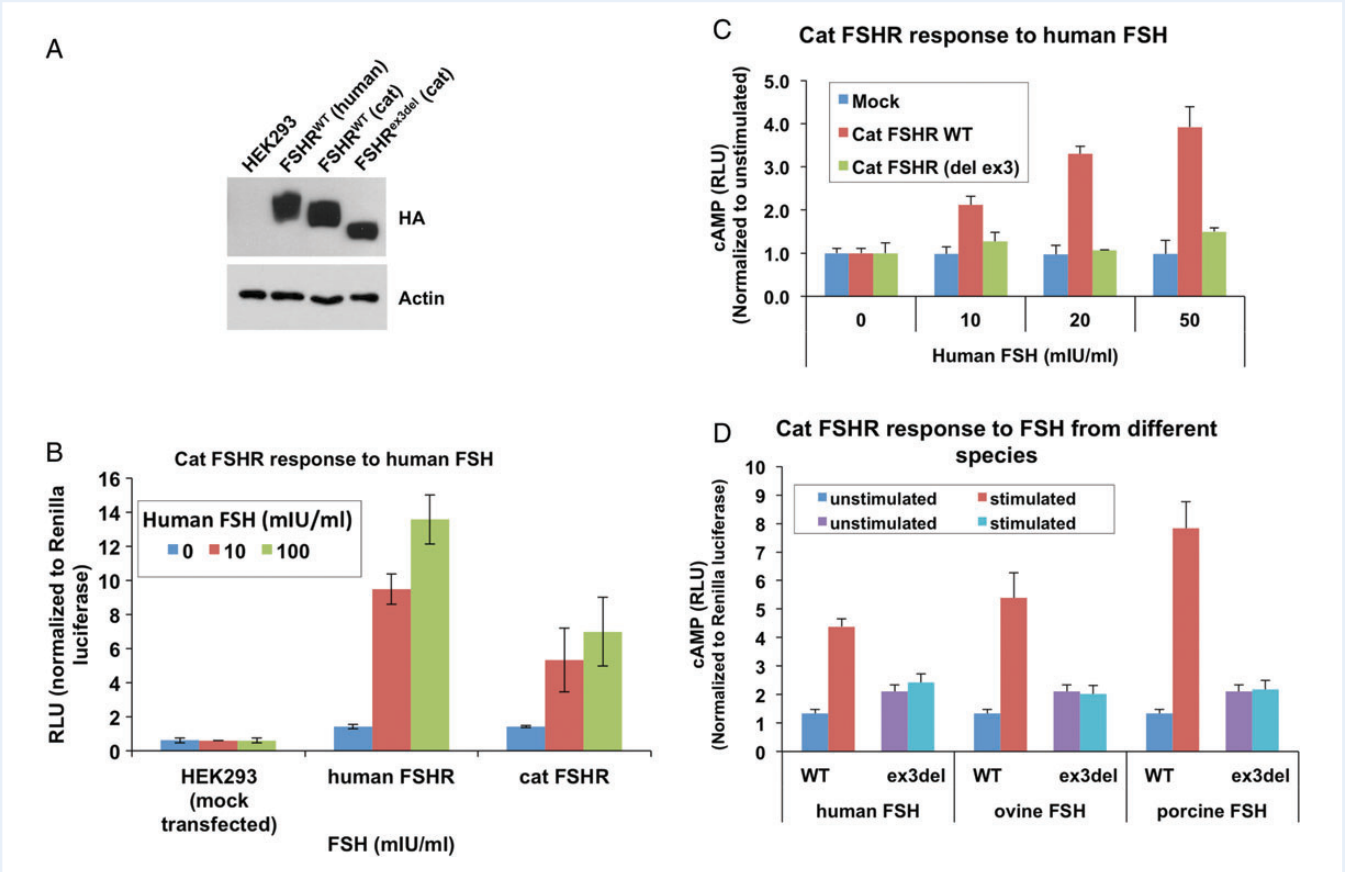


Figure 5 *In vitro* expression of cat FSHR^{ex3del} and response to FSH of different origins. (**A**) Western blot analysis of transient transfections with plasmids expressing human and cat FSHR^{WT} and cat FSHR^{ex3del} in HEK293 cells. Lane 1: Mock transfection of HEK293 cells. The receptor was tagged with HA as above. Top panel: hybridization with an anti-HA antibody. Bottom panel: a blot with the same lysates hybridized with an anti-β-actin antibody. (**B**) To measure response to human FSH, transient transfections in HEK293 cells were performed as in Fig. 4B. The cat FSHR^{WT} showed adequate response to human hormone. (**C**) The deleted cat FSHR was not able to increase cAMP production in response to human FSH. (**D**) Similar to human FSH, the deleted cat FSHR did not respond to ovine or porcine FSH. In contrast, the cat FSHR^{WT} responded well to FSH of different origin (human, 50 mIU/ml; ovine, 5 μg/ml; porcine, 2 U/ml).

Thus, heterodimers of the full-length and the splice variant receptor are likely to cause a dominant negative effect to the action of the WT receptor, as has been shown to occur in the case of dominant point mutations in *FSHR* (Zariñán *et al.*, 2010).

Two coding *FSHR* polymorphisms on exon 10 (p.Thr307Ala, p.Asn680Ser) have been extensively studied in relation to ovarian response during IVF treatment (reviewed in Mohiyiddeen and Nardo, 2010; Lalioti, 2011). The distribution of these variants in women seeking infertility treatments deviates from the expected frequency of the general population (Sudo *et al.*, 2002; Loutradis *et al.*, 2006; Achrekar *et al.*, 2009; Livshyts *et al.*, 2009). Although the original publications showed an association with basal serum FSH level, total FSH required for stimulation, serum estradiol levels at retrieval and the number of oocytes retrieved, subsequent studies failed to show an association or showed a very small effect (Perez Mayorga *et al.*, 2000; Sudo *et al.*, 2002; de Koning *et al.*, 2006; Jun *et al.*, 2006; Klinkert *et al.*, 2006; Loutradis *et al.*, 2006; Achrekar *et al.*, 2009; Overbeek *et al.*, 2009; Kuijper *et al.*, 2010; Mohiyiddeen *et al.*, 2013). The most plausible explanation is that these polymorphisms correlate with IVF results in patients of certain geographic origins but not in all. An alternative possibility is that they are important for particular subgroups of infertility patients and treatments. For example, patients homozygous for the less common allele (p.Ser680) have been shown to be resistant to clomiphene citrate (Overbeek *et al.*, 2009).

Although combined evidence does not support a robust association of the coding SNPs with IVF outcome, there is a strong indication for the involvement of *FSHR* genotypes in ovarian homeostasis. Genome-wide associations with a large number of polycystic ovarian syndrome patients have shown strong linkage of the disease to the genomic area that contain the FSHR and LH receptor genes (Chen *et al.*, 2011; Shi *et al.*, 2012; Zhao *et al.*, 2012; Mutharasan *et al.*, 2013). This is likely to be mediated through differences in expression or processing of the receptor resulting in alterations of the signaling cascade.

In the current study, no statistical difference was found in the clinical characteristics of patients under 35 years old carrying the FSHR^{ex2del} variant and patients without a variant, possibly due to the low total number of affected patients (Supplementary data, Table SIV). More studies are needed to investigate the role of the splicing variants described here in low responders and further delineate their clinical characteristics. However, collecting cells from low responders have

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three inherent difficulties: the great majority of low responder cycles are cancelled due to low follicle numbers and therefore cumulus cells do not become available for testing; FSHR expression is down-regulated by the hCG given to mature the oocytes (Ndiaye *et al.*, 2005); and fewer oocytes would yield fewer cumulus cells. Therefore, the method we optimized and used here (Fig. 2A and Materials and Methods) will be ideal for assessment of samples from low responders. In order to recruit more patients and not to limit our investigation to ICSI patients, we previously considered and tried to use granulosa cells isolated from the follicular fluid, rather than cumulus cells. However, we decided not to choose this approach for the following reasons. The follicular fluid in most cases contains a significant amount of blood and therefore some degree of coagulation occurs if not handled immediately. Separation of granulosa cells from red and white blood cells is achieved through a series of centrifugations, some of which include density gradients (for example, Percoll; (Ho *et al.*, 2006)). Finally, in order to completely separate granulosa cells from white blood cells it is preferable to put the cells in culture, in which case the granulosa attach to the dish, while the white blood cells remain in suspension (Ho *et al.*, 2006). However, extended culture of already luteinized granulosa cells will further down-regulate FSHR. This type of procedure would require a dedicated and trained person within the IVF lab, which was not feasible.

The recent technological advances for the analysis of the genome and the transcriptome, such as exome sequencing and RNA-seq have revolutionized our ability to identify mutations and polymorphisms, which are a direct cause of a disease rather than showing linkage to a genomic locus (Shah *et al.*, 2012; Clark *et al.*, 2013). The DNA or RNA amount necessary to perform this type of sequencing is low enough to allow analysis without amplification, and is compatible with the collection method we describe. Finding the genomic mutation or polymorphism will, in the future, permit us to test patients' DNA prior to IVF treatment and customize ovarian stimulation to an anticipated low response. An additional advantage is that mutations in other molecules can emerge as strong predictors of ovarian response.

The naturally occurring FSHR splicing variants in mammals and the splicing variants we have identified in low responders have two main implications. First, *FSHR* expression and function in mammals seems to be post-transcriptionally regulated. While the mechanisms controlling naturally occurring alternative splicing in mammals remain unknown, our finding that FSHR lacking exon 3 is unable to stimulate the main FSH signaling cascade suggest that these variants have significant implications on mammalian reproduction that should be further investigated. Secondly, aberrant FSHR splicing seems to be associated with low response and associated subfertility in women. Delineating the mechanism underlying loss of function of alternatively spliced FSHR in human may have significant implications for individualized diagnostic and therapeutic interventions.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

M.D.L. and E.S. designed the human study. C.K., S.G. and D.E. collected the samples and processed them for RNA extraction. C.K., O.G.-K. and T.G. extracted RNA, made cDNA and performed RT–PCR and sequencing. M.E., M.O., A.E. and E.S. screened patients for suitability for the study, reviewed history, decoded patient information. M.D.L., D.S. and P.C. designed the study of the cat FSHR. M.D.L., R.J.H. and T.G. performed the cat FSHR experiments. C.K., A.U., E.S. and M.D.L. analyzed data and wrote the manuscript. All authors reviewed and revised the manuscript.

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Conflict of interest

All authors have nothing to disclose.

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