Targeted expression of human FSH receptor Asp567Gly mutant mRNA in testis of transgenic mice: role of human FSH receptor promoter

Verena Nordhoff[1], Jörg Gromoll[1], Luca Foppiani[1*], C. Marc Luetjens[1], Stefan Schlatt[1], Elena Kostova[1], Ilpo Huhtaniemi[2#], Eberhard Nieschlag[1], Manuela Simoni[1]

[1]*Institute of Reproductive Medicine, University of Mnster, D-48149 Mnster, Germany*
[2]*Department of Physiology, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland*

Asian J Androl 2003 Dec; 5: 267-275

Keywords: **transgenic mouse; follicle-stimulating hormone receptor; promoter; activating mutation; Sertoli cells**

Abstract

Aim: To specifically express the Asp567Gly human follicle-stimulating hormone receptor (FSHR) under the control of its promoter to evaluate the phenotypic consequences in the presence of normal pituitary function. **Methods**: We produced transgenic mice overexpressing the Asp567Gly human FSHR under the control of a 1.5kb 5'-flanking region fragment of its promoter. **Results**: Mice were phenotypically normal and fertile. In males, mRNA could be detected in the testis and the brain, indicating that the 1.5kb promoter fragment drives expression not only in the gonads. The testis weight/body weight ratio and the testosterone levels in transgenic and non-transgenic littermates were similar. By in situ hybridisation we found that the transgenic FSHR was highly expressed in Sertoli cells, spermatocytes and round spermatids. However, a radioligand receptor assay failed to show a significant difference in total FSHR binding sites in testis homogenates of transgenic and wild type animals, suggesting that the transgenic FSHR is probably not translated into functional receptor protein. **Conclusion:** A 1.5kb 5'-region of the human FSHR drives mRNA expression of the transgene in the testis but leads to ectopic expression in germ cells and in the brain. No phenotypic consequences could be documented due to the lack of protein expression

1 Introduction

Together with testosterone, follicle-stimulating hormone (FSH) plays an important role in the onset and maintenance of spermatogenesis [1]. In the male, FSH acts specifically and solely on Sertoli cells [2]. FSH binds to the FSH receptor (FSHR) and initiates the production of the second messenger cAMP. The molecular events following the binding of FSH to its receptor and their role in the control of spermatogenesis are not exactly known.

In the primate impaired spermatogenesis may be a result of decreased FSH action [3]. Naturally occurring inactivating mutations of the FSHR leading to reduced follicular maturation and spermatogenesis have been described [4]. However, in mice in which the FSHR was disrupted spermatogenesis was maintained and males were still fertile despite a lower testis size in comparison with the wild types [5]. Male mice with a disrupted FSH b-subunit also had a reduced testis size but were fertile. On the other hand, overexpression of FSH in hpg mice, a model for "pure" FSH action, led to a partial induction of spermatogenesis in the complete absence of LH/testosterone secretion [6], suggesting an important role for FSH also in this species.

We described an activating mutation of the FSHR in a patient with normal spermatogenesis in the absence of LH and FSH because of a hypophysectomy due to a pituitary tumor [7]. As a remarkable exception among hypophysectomized patients, this patient had normal spermatogenesis under testosterone treatment. To explain this phenotype we hypothesised a constitutively activated FSHR which was active in the absence of FSH. In this patient we demonstrated a Asp567Gly transition in exon 10 of the FSHR [7], leading to slight ligand-independent receptor activation in transiently transfected Sertoli cells [2].

In another study we could show that when the Asp567Gly FSHR driven by a 1.4 kb fragment of the rat ABP promoter was expressed in gonadotropin-deficient transgenic mice, it resulted in ligand-independent FSH-like activity in the testis [6, 8].

No other activating mutations of the FSHR have been described in patients for a long time [9]. Very recently two activating mutations of the FSHR were described which resulted in "hyperreactio luteinalis" during pregnancy [10, 11].

The present work was undertaken to analyse the clinical consequences of an activating FSHR mutation *in vivo* when pituitary function is normal. For this purpose we generated transgenic mice overexpressing the human Asp567Gly FSHR under the control of 1.5 kb of the human FSHR promoter.

2 Materials and methods

2.1 DNA construct

In serveral cloning steps a minigene construct including intronic sequences was produced (Figure 1). The mutation Asp567Gly was introduced into the wild type cDNA of the human FSHR by *in vitro* mutagenesis. This cDNA of the mutated human FSHR (mutFSHR) in pBluescript SK(-) (Stratagene; pBS) and a genomic clone encompassing Exon 2 flanked by intronic sequences of intron 1 and intron 2 were digested with Eco0190 I and Kpn I. The excised Exon 2 with intronic sequences replaced Exon 1-2 of the mutated FSHR. This intermediate construct containing intronic sequences of intron 1 and Exon 2-10 was cut out by digestion with SacII and Kpn I and cloned upstream into a linearised (Sma I) pBS vector containing the SV40 poly A site. In a further step the 1.5 kb 5'-region of the human FSHR promoter, Exon 1 and 3'-sequences of intron 1 were cut out from another genomic clone by digestion with Sac I and EcoR V and ligated in front of the linearised (Not I) intermediate construct to produce the final construct containing the 1.5 kb 5'-region of the human FSHR, Exon 1, sequences of intron 1, Exon 2-10 (Exon 10 containing the mutation) and the SV40 poly A site (Figure 1).

Figure 1. Schematic picture of human mutFSHR minigene construct under control of human FSHR promoter. Exon 10 includes activating mutation Asp567Gly. (bp: base pairs, SV: simian virus, h FSHR: human FSHR).

2.2 DNA purification for pronuclear injection

The construct was released from the vector pBS (SK-) by digestion with Sac II and Kpn I and the desired band was cut out from a 1 % agarose gel. The DNA was purified by polymerase chain reaction (PCR) Template Purification Kit (Roche) and then precipitated with 100 % ethanol. After several washing steps with 70 % ethanol, the DNA was resolved in a sterile Tris-EDTA-solution according to Hogan *et al* [13]. The DNA was stored at -20 until injection.

2.3 Production of transgenic mice

Animal experiments were performed according to the German law on Care and Management of Laboratory Animals. Transgenic mice were produced according to the methods described by Hogan *et al* [12]. The trans-gene was injected into fertilised eggs from C57BL/6/DBA2 C57BL/6 hybrid mice. Two transgenic founders with the human construct, a male and a female were produced. Transgene insertion of the construct was detected by a PCR with specific human primers, a sense primer binding to the intronic sequences 5' of exon 2 (5'-TCACTA-GTGTAGACAGGATG-3' and a reverse primer binding to exon 2-3 (5'-GAGATCTCTATTTTCTCCAGG-3' leading to a band of 272 bp. As DNA control a 202 bp fragment of exon 10 of the mouse FSHR was amplified by PCR with primer mouse 1 (5'-CAATTCTTGTGC-CAATCCTTTC-3' and primer mouse 2 (5'-CAAGCA-CGTAACTATTGGTG-3'. Lines were established by crossing the founders with C57BL/6 male or female mice.

2.4 RNA

Mice were killed at the age of 7 days, 14 days, 21 days, 35 days and 56 days. One testis and other organs were snap frozen and stored at -80 . RNA was isolated by the Ultraspec method (Biotecx Laboratories) according to manufacturers' instructions, dissolved in DEPC-water and stored frozen at -80 until use.

2.5 RT-PCR

RNA (10 g) was incubated with 200 IU Moloney murine Leukemia Virus reverse transcriptase (Promega), 4 L 5buffer, 1 L 40 IU/L RNAsin, 1 L 10ng/L primer human/mouse Exon 7 reverse (5'-GCACAGTTG-TGTATTTCTTGAATCCC-3', 1 L 10 ng/L primer actin reverse (5'-GAGCAATGATCTTGATCTTC-3', 2 L 100 mmol/L dNTP's in a final volume of 20 L at 37 for 45 min ~ 60 min. With 5 L (FSHR) of this reverse transcribed cDNA a PCR was performed, using 1 L 100 ng/L primer human Exon 1 sense (5'-CAGGGTTTTTCTCTGCCAAGAG-3', 1 L 100 ng/L human/mouse Exon 7 reverse (5'-GCACAGTTGTGTGATTTCTTGAATCCC-3', 1 L 100 mmol/L dNTP's, 1.5 L 25 mmol/L MgCl, 2.5 L 10PCR buffer and 5IU Polymerase (Promega). As the control 2 LcDNA was used in a PCR with primers for actin sense (5'-GATGACCCAGATCATGTTTG-3' and actin reverse (5'-GAGCAATGATCTTGATCTTC-3'. PCR was run at 94 for 2 min, 35 cycles at 94 for 40 sec, 55 for 40 sec, 72 for 1 min and a final 72 for 10 min. Samples were analysed on a 1 % agarose gel.

2.6 Northern blot

Isolated RNA was heat denatured and equal amounts (20 g) were run on a 1 % agarose gel with ethidium bromide in a loading dye, blotted and fixed on nitro-cellulose. For day 21 two and for day 7 three tissue samples were pooled. For producing a probe, 1 g FSHR cDNA was incubated with 6 L 5buffer, 4 L 2.5 mmol/L dNTP's, 2.4 L 100 mol/L CTP, 3 L 100 mmol/L DTT, 1.5 L RNAsin (40 IU/L), 5 L ^{32}P-CTP (50 C) and 1.5 L T7 15 IU RNA polymerase at 37 for 1 h and with additional 1.5 L 15 IU T7 RNA polymerase for 1 h. To this mixture 70 L DEPC water and 2 L DNAse I (1IU) was added and incubated for 30 min at 37 . It was precipitated with 50 L 7.5 mol/L ammonium acetate, 4 L tRNA and 400 L 100 % ethanol, centrifuged and dissolved in 50 L DEPC water. This probe showed 310^6 counts per L. After a prehybridisation at 50 for 1 h the blot was hybridised with 6 L of the probe at 55 overnight. The blot was washed twice with 2SSC containing 0.1 % SDS 10 min ~ 20 min at RT and twice with 0.1SSC containing 0.1 %SDS 30 min at 55 . Detection of specific bands was done by Phosphor Imager (Storm).

2.7 Histology

Testes were fixed in Bouin's solution for 10 h ~ 24 h and washed in 70 % ethanol. Slices of 4 m ~ 5 m were stained with PAS and morphometrical histological analysis was performed.

2.8 In situ hybridisation

The in situ hybridisation was performed as described [13]. The probe for hybridisation was prepared by incubation of 1 g linearised plasmid with 4 L 5trans-criptionbuffer, 2 L 10DIG RNA labelling mix (Roche), 2 L 100 mmol/L DTT, 0.75 L RNAsin (40 IU/L) and 1.5 L T7 15 IU RNA polymerase (Promega) for 1 h at 37 and with additional 1 L 15 IU RNA polymerase for another 1 h. With 5 L 1 IU DNase I the mixture was incubated for another 20min. Reaction was stopped by adding 2 L 0.2 mol/L EDTA (pH 8). Purification was carried out with "High pure PCR purification kit" (Roche) and eluted with 70 L DEPC water (pH 8). Bouin-fixed tissue was cut into 5 m ~ 7 m sections, transferred onto slides and dried overnight at 42 . Sections were deparaffinised by 15 min Paraclear I and 5 min Paraclear II, dehydrated through an alcohol series made with DEPC water (2100 %, 195 %, 180 % and 165 % ethanol), denaturated with 0.2 N HCl in 1PBS for 20 min and incubated in 2SSC at 70 for 15 min. After washing sections with 1PBS for 5min they were permeabilised with 2 g/mL proteinase K for 12 min at 37 in 50 mmol/L Tris-HCl (pH 8) and 5 mmol/L EDTA (pH 8). Reaction was stopped by 10 min incubation at 4 with 0.2 % glycine 1PBS and then post-fixed for 5 min at 4 with 4 % paraformaldehyde in 1PBS. After washing 25 min with 1PBS the sections were acetylated 25 min with 0.1 mol/L trietholamine containing 0.25 % acetic anhydride, dipped in 1PBS and prehybridised for 1 h ~ 2 h at 43 in a humid chamber. Hybridisation was performed with 50 ng of Dig-labelled RNA probe (after 5 min at 70) overnight at 43 . After washing 215 min with 2SSC and 215 min with 1SSC, single-stranded unbound RNA was digested for 30 min at 37 in NTE buffer (500 mmol/L NaCl, 10 mmol/L Tris, pH 8, 5 mmol/L EDTA, pH 8) with 20 g/mL RNase. After this the sections were washed 230 min with 0.1SSC. For immunological detection the sections were washed 35 min with 1TBS and incubated for 30 min with blocking solution (20 % sheep serum in 1TBS) and then the solution was replaced by 1TBS and incubated in a humid chamber for 2 h at room temperature with an anti DIG-AP-Ab solution. After washing sections 3 10 min with 1TBS they were incubated in a humid chamber for several hours under drops of NTB/BCIP substrate solution (Boehringer) containing 1.5 mmol/L levamisol. After reaching the desired staining the reaction was stopped by immersing in ddH_2O. Sections were counter-stained with Mayer's haemalaun for 2 s ~ 5 s and then washed 10 min ~ 15 min in running tap water.

2.9 Radioligand binding assay

FSHR binding experiments were carried out as described [14]. Binding of ^{125}I-FSH (130-146 Ci/g) to whole testis homogenates of wild type (WT, *n*=20) and transgenic mice (*n*=6) was studied in isotonic HEPES-buffered Krebs Ringer buffer (4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 2.5mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 200 mmol/L sucrose and 25 mmol/L HEPES, pH 7.4) containing 0.1 % (w/v) BSA. In saturation experiments homogenates were incubated with increasing concentrations of tracer (9.4 pmol ~ 150 pmol) in the presence or absence of unlabeled FSH (Fertinorm; 1 IU/250 L) at 300 fold molar excess. At the end of the equilibration period the buffer was aspirated and the homogenates were washed twice with ice-cold buffer. The homogenates were subsequently dissolved in 1 N NaOH and total homogenate associated radioactivity (the sum of surface-bound and internalised radioactivity) was measured by LKB Wallac 1277 automatic gamma counter. Values for the apparent affinity constant and the number of binding sites were calculated by least square analysis using the GraphPad PrismTM, version 2.0 computer programme.

2.10 Serum testosterone

Blood was collected and serum was isolated by centrifugation. Serum testosterone was measured by radioimmunoassay [15].

2.11 Statistics

Testis weight / body weight ratios and testosterone values were analysed using t-test, Kruskal-Wallis test and Dunn's Multiple Comparison test. $P<0.05$ was considered significant.

3 Results

Two transgenic founders, a male and a female, were produced. Only the male founder transmitted the transgene to the offspring and generated a line of trans-genic mice. The transgene transmission was according to the Mendelian rules with a normal sex ratio.

The transgene expression in the testis and other organs was detected by means of RT-PCR (Figure 2). The isolated mRNA was transcribed into cDNA with a reverse primer binding in Exon 7. This primer recognised the human and the mouse transgene and also the endogenous mouse FSHR, but had a higher specificity for the transgenes. A PCR with primers in human Exon 1 and human/mouse Exon 7 revealed a product of 478 bp in both transgenic and non-transgenic mice, but showed a higher expression in the transgenic mice. No signal could be detected in kidney and liver of transgenic and WT mice. The expression of the transgene was further analysed by Northern blot with a probe against Exon 9-10 of the human FSHR of testis and other organ tissues from transgenic and WT mice (Figure 3). High expression of the transgene was detected in the testis of the transgenic lines. Interestingly, faint expression was also detected in the brain of transgenic mice after long exposure. No expression could be detected in ovaries of female transgenic mice (data not shown).

Figure 2. RT-PCR for detection of transgene. Upper part shows strong 478bp band of human FSHR in testis of a mouse with human promoter minigene (tg) compared to faint band of exogenous FSHR of a wild type mouse (wt). Other tissues e.g. kidney are negative in both wild type and transgenic mice. Lower part shows actin control for intact DNA. (Marker: lambda Hind III).

Figure 3. Detection of hFSHR transgene mRNA in different mouse tissues in a northern blot. Testis, brain and kidney of one transgenic and one wild type mouse hybridised with a probe against human FSHR. Human promoter mutFSHR minigene with expected size of 2.5 kb (in relation to 28S and 18S RNA) was detected in testis and also in brain of transgenic mice. (wt: wild type, tg: transgenic mice with the human promoter mutFSHR transgene).

To test whether the transgene was also expressed at earlier time points of development, mice were killed at the age of 7 days, 21 days and 56 days and one testis was analysed by Northern blot (Figure 4). Transgenic mice from the age of 21 days onwards exhibited detectable signals of the expected size. Expression increased from day 21 to day 56, concomitantly with the achievement of complete spermatogenesis.

Figure 4. Age-dependent expression in mice with human promoter minigene (tg) in comparison to wild type mice (wt). For analysis, 3 animals at day 7, 2 animals at day 21 and one animal at day 56 were used. Expression could first be detected at day 21 and increased expression was found at day 56 in transgenic mice with human promoter minigene.

The cellular localisation of the transgene mRNA was analysed by in situ hybridisation. Figure 5 shows that the transgene was expressed not only in Sertoli cells but also in germ cells up to spermatocytes and round sperma-tids.

Figure 5. *In situ* hybridisation for localisation of human FSHR transgenic mRNA in wild type (wt) and transgenic mice with the human promoter minigene (tg). The sense control reaction was applied on left side and antisense probe applied on right side. *In situ* hybridisation with transgenic mice shows positive staining in Sertoli cells and germ cells up to spermatocytes and round spermatids. (scale bar: 50 m).

These data suggest that a 1.5 kb fragment of the 5?flanking region of the human FSHR is sufficient to direct mRNA expression of a transgenic receptor in the testis but not exclusively in the Sertoli cells.

To evaluate the effects of transgene expression on testicular function, testis weight, serum testosterone and testicular histology were analysed. Testis weight (Table 1) and serum testosterone levels (Table 2) were not significantly different between WT and transgenic mice.

Table 1. Testis weight/body weight ratio in transgenic and wild type mice (meanSEM).

Age (days)	Transgenic mice with human promoter minigene (n)	Wild type mice (n)
7	2.480.09 (12)	2.580.09 (34)
14	2.960.09 (10)	3.770.37 (8)
21	4.350.28 (7)	5.000.34 (7)
35	7.260.28 (6)	7.310.26 (10)
56	7.760.24 (3)	8.540.38 (10)

Table 2. Serum testosterone levels of transgenic and wild type mice of different ages (meanSEM nmol/L).

Age (days)	Transgenic mice with the human promoter minigene (n)	Wild type mice (n)
14	1.800.23 (9)	1.650.21 (8)
21	1.310.17 (6)	1.680.25 (7)
35	3.501.60 (5)	2.230.58 (10)
56	5.724.13 (3)	5.972.83 (10)

Similarly, testicular histology evaluated at 7 days, 14 days, 21 days, 35 days and 56 days of age revealed no apparent difference between the transgenic and non-transgenic mice (data not shown).

Because of the high expression level and the absence of functional consequences thereof, we assessed the concentration of FSH binding sites in the testes of WT and transgenic mice by radioligand receptor assay. Transgenic mice had only slightly increased FSH binding site concentrations [$(1.130.01)10^{11}$ *vs* $(1.080.08)10^{11}$ sites/mg protein] suggesting that most if not all of the highly expressed mutFSHR mRNA is probably not translated into a functional protein.

4 Discussion

The aim of the present work was to analyse the phenotype *in vivo* in the presence of a normal hypothalamic-pituitary-gonadal axis of a previously described human FSHR mutant showing ligand-independent activation when transiently expressed in a Sertoli cell line [2]. In order to express specifically the activated FSHR in the testis we chose the promoter of the human FSHR. We succeeded in generating transgenic mice but possibly due to problematic insertion of the transgene we could only analyze one transgenic line. Male mice of this line successfully produced mRNA of the Asp567Gly mutFSHR under control of the human FSHR promoter in the testis. However, no increased FSH binding could be documented in transgenic mice, suggesting that the large amounts of transgenic mRNA most probably are not translated into a functional protein. Alternatively the unchanged FSH binding to testis homogenates in transgenic mice could reflect autoregulation of net FSH receptor protein production in Sertoli cells perhaps due to competition for limited numbers of membrane insertion sites. In a parallel experiment our basic minigene construct driven by the rat ABP promoter was used to generate transgenic mice on a hypogonadal background (*hpg* mice) and resulted in transgenic mice expressing the mutated FSHR protein solely in the testis [8]. The rat ABP promoter is Sertoli cell-specific and highly active [16]. The findings of Hayworth *et al* [8] suggest that our minigene construct itself is functional because it is expressed as mRNA and is also translated into a functional protein. The lack of protein expression and the ectopic mRNA espression in this study seems to be the result of the human FSHR promoter which might be not suitable for this purpose.

The 1.5 kb fragment of the human FSHR promoter was chosen to achieve specific expression in the Sertoli cells based on our previous data. In a chloramphenicol-acetyltransferase reporter gene assay (CAT-assay) shorter fragments of 225 bp length of the 5'-region of the human FSHR showed high but not specific expression of the reporter gene in several cell lines. The longest fusion construct with a 1.5kb fragment of the 5'-region of the FSHR resulted in a low but more specific expression in gonadal cell lines [17]. Therefore this 1.5 kb fragment was chosen for our minigene construct. The results of this study show that this promoter fragment can direct mRNA expression in Sertoli cells but also leads to high ectopic expression in germ cells and to faint expression in the brain.

Based on previous *in vitro* data we expected specific expression [17]. However, high levels of expression in Sertoli cells could not be anticipated since the mechanisms regulating endogenous FSHR expression should control the promoter of the transgene as well. Therefore, the faint expression in brain, the high expression in germ cells and the lack of expression in the ovary were surprising. Meanwhile, others made similar observations by using fragments of two different lengths (5000 bp and 100 bp) of the 5'-flanking region of the rat FSHR promoter fused to Cre recombinase and the bovine GH gene [18]. In addition to expression in the testis, all different transgenic mouse lines with the long promoter fragment showed a marked expression in the brain. Ectopic expression in other tissues was occasionally observed in some lines with both long and short promoter fragments [18]. In most lines based on the long promoter fusion constructs, transgene expression in the ovary was found, while only few lines with the short promoter showed expression in the ovary. The authors conclude that the first 100 bp of the promoter are sufficient for expression in the testis but lack some sequences necessary for expression in the ovary [18]. By isolating Sertoli or germ cells of 30 day old transgenic mice with the short promoter construct, the authors found that only germ cells exhibited high levels of the reporter mRNA, while the Sertoli cell fraction showed only little expression which may be due to contaminating germ cells [18]. This is in contrast to our finding where the mutFSHR mRNA was found in Sertoli cells at day 56. This difference could be due to the different promoter length in both constructs or might be a species-related effect (human *vs.* rat).

Interestingly, constructs with the murine luteinizing hormone receptor (LHR) promoter led to similar findings [19]. A 2.1 kb part of the LHR promoter could direct specific expression of b-galactosidase (-GAL) in adult Leydig cells but also showed ectopic expression in elongating spermatids and in the brain. No expression was found in ovaries of transgenic females. The authors hypothesise that cis-regulatory elements outside the 2.1 kb promoter sequence are necessary for directing expression in the ovary [19]. Yet extending the LHR promoter to up to 7.4 kb showed similar expression in Leydig cells and in elongating spermatids [20]. Expression in ovarian granulosa or luteal cells could be detected only in one line of transgenic mice. This line also exhibited high expression in the brain and other tissues [20].

Taken together, our present results and the results of the literature show that even quite extended fragments of the 5'-region of the gonadotropin receptor promoters are not sufficient per se to specifically direct the expression of the FSHR and LHR to the cells that physiologically host them. The reason for this remains unclear.

The high transcription rate of the transgene in germ cells is peculiar. It is known that the testis and especially the germ cells have high transcriptional activity, as shown in several genes of different function in different species. Possibly the transgene is transcribed within the mass of other genes. An alternative explanation for the frequent occurrence of ectopic expression in germ cells could be an effect of gap junctions and intercellular bridges by which the mRNA transcribed in Sertoli cells could be transferred to these germ cells [21].

Although highly expressed in germ cells, the Asp567Gly receptor was not translated into a functional protein. Since immunological assessment of the FSHR protein is hampered by the lack of suitable species-specific antibodies, we analysed protein expression indirectly by binding experiments. Our radioligand binding data suggest that both transgenic and non-transgenic mice have nearly identical receptor numbers in the whole testis. Similar to the present results, using transgenic mice with a rat FSHR promoter b-GAL fusion gene expression was found in the testis but protein could never be detected by histochemical methods [22]. The authors ascribed this to the weakness of the promoter and to the limited sensitivity of the -GAL method, or possibly, to improper translation [22]. Since the same mutFHSR expressed under the control of the rat ABP promoter was shown to be functional on the *hpg* background [8], we exclude the possibility that the minigene was not functional and conclude that the lack of transgenic protein expression in the transgenic mice described here is related to the fragment of the human FSHR promoter used and not to improper translation. It seems that this promoter is not sufficient to produce a mRNA that can be translated into functional protein. Alternatively, it could be translated into protein only in the Sertoli cells and its expression could be regulated as that of the endogenous receptor. This would result in no net change in the receptor number.

Recently it has been described that small interfering RNAs (siRNAs) can cause the degradation of homologous endogenous mRNA and create a loss-of-function phenotype among different eukaryotic species. This technique also called RNA interference (RNAi) is used to produce transgenic mice where the target gene is stably silenced. It seems that small RNA can operate as regulator of gene expression not only at a transcriptional but also on a post-transcriptional level. A similar mechanism might lead to an unwanted lack of protein translation in transgenic mice which results in no phenotypic changes.

In summary, we showed that a 1.5 kb fragment of the 5?flanking region of the human FSHR promoter is sufficient for mRNA expression in the testis but not only in Sertoli cells and that high levels of ectopically expressed mRNA most probably remain untranslated. Consequently no phenotypic consequences could be documented. No expression was detected in the ovary. In conclusion, 1.5 kb of the human FSHR promoter are not sufficient to direct the appropriate expression of the FSHR in the testis and in the ovary.

Acknowledgements

The work was supported by the DFG Ni 130-15 and the Deutscher Akademischer Austauschdienst (DAAD). The authors would like to thank Elisabeth Pekel, Sabine Borchert, Bettina Khan and Reinhild Sandhowe-Klaverkamp for their excellent technical assistance. Animal care of Martin Heuermann and Gnther Stelke is also gratefully acknowledged. Dr. K. Gopalkrishnan helped in the histological evaluation and we especially thank Susan Nieschlag M.A. for language editing.

References

[1] Simoni M, Weinbauer GF, Gromoll J, Nieschlag E. Role of FSH in male gonadal function. Ann Endocrinol (Paris) 1999; 60: 102-6.
[2] Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr Rev 1997; 18: 739-73.
[3] Plant TM, Marshall GR. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocr Rev 2001; 22: 764-86.
[4] Tapanainen JS, Aittomäki K, Min J, Vaskivuo T, Huhtaniemi IT. Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. Nat Genet 1997; 15: 205-6.
[5] Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, *et al.* Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 1998; 95: 13612-7.
[6] Allan CM, Haywood M, Swaraj S, Spaliviero J, Koch A, Jimenez M, *et al.* A novel transgenic model to characterize the specific effects of follicle-stimulating hormone on gonadal physiology in the absence of luteinizing hormone actions. Endocrinology 2001; 142: 2213-20.
[7] Gromoll J, Simoni M, Nieschlag E. An activating mutation of the follicle-stimulating hormone receptor autonomously sustains spermatogenesis in a hypophysectomized man. J Clin Endocrinol Metab 1996; 81: 1367-70.
[8] Haywood M, Tymchenko N, Spaliviero J, Koch A, Jimenez M, Gromoll J, *et al.* An activated human follicle-stimulating hormone (FSH) receptor stimulates FSH-like activity in gonadotropin-deficient transgenic mice. Mol Endocrinology 2002; 16: 2582-91.
[9] Nordhoff V, Gromoll J, Simoni M. Constitutively active mutations of G protein-coupled receptors: the case of the human luteinizing hormone and follicle-stimulating hormone receptors. Arch Med Res 1999; 30: 501-9.
[10] Vasseur C, Rodien P, Beau I, Desroches A, Grard C, de Poncheville L, *et al.* A chorionic gonadotropin-sensitive mutation in the follicle-stimulating hormone receptor as a cause of familial gestational spontaneous ovarian hyperstimulation syndrome. N Engl J Med 2003; 349: 753-9.
[11] Smits G, Olatunbosun O, Delbaere A, Pierson R, Vassart G, Costagliola S. Ovarian hyperstimulation syndrome due to a mutation in the follicle-stimulating hormone receptor. N Engl J Med 2003; 349: 760-6.
[12] Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the mouse embryo - A laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1994.
[13] Gromoll J, Wessels J, Rosiepen G, Brinkworth MH, Weinbauer GF. Expression of mitotic cyclin B1 is not confined to proliferating cells in the rat testis. Biol Reprod 1997; 57: 1312-9.
[14] van Loenen HJ, Flinterman JF, Rommerts FF. Follicle-stimulating hormone stimulates rat Sertoli cells via relatively low affinity binding sites. Endocrine 1994; 2: 1023-9.
[15] Chandolia RK, Weinbauer GF, Behre HM, Nieschlag E. Evaluation of a peripherally selective antiandrogen (Casodex) as a tool for studying the relationship between testosterone and spermatogenesis in the rat. J Steroid Biochem Mol Biol 1991; 38: 367-75.
[16] Larriba S, Esteban C, Toran N, Gerard A, Audi L, Gerard H, *et al.* Androgen binding protein is tissue-specifically expressed and biologically active in transgenic mice. J Steroid Biochem Mol Biol 1995; 53: 573-8.
[17] Gromoll J, Dankbar B, Gudermann T. Characterization of the 5' flanking region of the human follicle-stimulating hormone receptor gene. Mol Cell Endocrinol 1994; 102: 93-102.
[18] Heckert LL, Sawadogo M, Daggett MA, Chen JK. The USF proteins regulate transcription of the follicle-stimulating hormone receptor but are insufficient for cell-specific expression. Mol Endocrinol 2000; 14: 1836-48.
[19] Hämäläinen T, Poutanen M, Huhtaniemi I. Age- and sex-specific promoter function of a 2-kilobase 5'-flanking sequence of the murine luteinizing hormone receptor gene in transgenic mice. Endocrinology 1999; 140: 5322-9.
[20] Hämäläinen T, Poutanen M, Huhtaniemi I. Promoter function of different length of the murine luteinizing hormone receptor gene 5'-flanking region in transfected gonadal cells and in transgenic mice. Endocrinology 2001; 142: 2427-34.
[21] Hecht NB. Intracellular and intercellular transport of many germ cell mRNAs is mediated by the DNA- and RNA-binding protein, testis-brain-RNA-binding protein (TB-RBP). Mol Reprod Dev 2000; 56: 252-3.
[22] Linder CC, Heckert LL, Goetz TL, Griswold MD. Follicle-stimulating hormone receptor gene promoter activity. Endocrine 1994; 2: 957-66.

Correspondence to: Prof. Dr. E. Nieschlag, FRCP, Institute of Reproductive Medicine, University of Mnster, Domagkstr. 11, D-48149 Mnster, Germany.
Tel: +49-251-835 6096, Fax: +49-251-835 6093
E-mail: nieschl@uni-muenster.de
Received 2003-07-18 Accepted 2003-09-29
*Current address: Dip. di Scienze Endocrinologiche e Metaboliche (DiSEM), University of Genova, I-16132 Genova, Italy.
#Current address: Institute of Reproductive and Developmental Biology (IRDB), Imperial College London, Du Cane Road, London W12 0NN, U.K.