大韓不妊學會雜誌: 第25卷 第2號 1998
Kor. J. Fertil. Steril., Vol. 25, No. 2, 1998, 8

Expression of Luteinizing Hormone (LH) Subunit Genes in the Rat Ovary

Sung Ho Lee*

Department of Biology, College of Natural Sciences, Sangmyung University, Seoul 110-743

흰쥐 난소에서의 Luteinizing Hormone (LH) Subunit 유전자 발현

상명대학교 자연과학대학 생물학과

이 성 호*

= 국문초록 =

흰쥐 난소에서의 Luteinizing hormone (LH) subunit 유전자 발현과 LH polypeptide의 존재를 조사하였다. 이를 위해 LH subunit들에 대한 역전사 중합효소연쇄반응 (RT-PCR)을 시행하였고, 난소내 LH 함량을 방사면역측정법으로 정량하였다. 뇌하수체와 정소에서 공통적으로 존재하는 LH-β subunit (LH_β)의 exon에 해당되는 primer를 사용하여 RT-PCR을 시행한 결과 흰쥐 난소에서도 뇌하수체, 정소와 같이 306 bp band가 확인되었고, 정소특이적인 exon에 해당되는 primer를 사용한 결과 정소와 난소에서 예상대로 428 bp band가 검출되었다. 또한 LH, FSH, TSH 그리고 hCG에서 공통적으로 발현되는 common α-subunit (C_α)의 전사물질도 PCR에 의해 증폭되었다. 방사면역측정법에서는 LH standard curve와 난소추출물을 사용한 curve가 동일하게 sigmoid 형태를 보임으로서 흰쥐 난소내에 immunoreactive LH가 존재함이 증명되었다. 인위적으로 성적인 성숙을 유도한 PMSG 주사 동물에서 혈중 LH 수준은 주사 후 48시간에 preovulatory LH surge와 유사한 최고 수준을 나타냈으나, 난소내 LH 함량의 경우 주사 24시간 후부터 급격히 감소하여 주사 48, 72시간군까지도 낮은 수준이 유지되었다. 이 결과는 흰쥐 난소의 LH가 생리적으로 조절되고 그 조절방식이 뇌하수체에서와는 다를 가능성을 시사하는 것이다. 본 연구는 흰쥐의 난소에서 LH과 유전자가 발현됨을 최초로 보고한 것이며, LH의 경우 내분비적 경로 (endocrine; 뇌하수체로 부터의 LH)외에도 국부적 경로 (autocrine이나 paracrine; 난소내에서 합성되는 LH)를 통해 난소의 생리와 기능 조절을 담당함을 시사한다.

Key Words: LH, Gene expression, Rat ovary, RT-PCR, Tissue specificity, Intraovarian factor

INTRODUCTION

The pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are synthesized and secreted by gonadotropes in anterior pituitary, and they play a pivotal role in both male and female reproduction. LH and FSH belong together with other pituitary hormone, thyroid stimulating hormone (TSH), and placental chorionic gonadotropin (CG) to the family of glycoprotein hormones (Pierce & Parsons, 1981; Albanese *et al.*, 1996). Each hormone consists of non-covalently linked α- and β-subunits, which are encoded by separated genes (Ryan *et al.*, 1988).

*To whom correspondence should be addressed.
Address: Department of Biology, College of Natural Sciences, Sangmyung University, Hongji-dong 7, Jongrho-gu, Seoul 110-743, Fax: (02) 396-6133; Tel: (02) 287-5139
본 연구는 1998년도 한국 과학재단 핵심전문연구과제(과제번호 981-0506-031-2)의 지원으로 이루어졌슴

In mammals, the α-subunit ($C_α$) is encoded by a single gene and common to all of the glycoprotein hormones while the unique β-subunits encoding by different genes provide the biological specificity of each hormone (Fiddes & Goodman, 1981; Ryan *et al.*, 1988).

Though the anterior pituitary had been thought to be the only source of LH for a long time, recent studies from the Finnish research group clearly demonstrated the novel expression of LH β-subunit ($LH_β$) genes in the rat testis (Zhang *et al.*, 1995a). Three $LH_β$ clones with length of 3.2, 2.4 and 0.86 Kb were identified in a rat testicular cDNA library, and the nucleotide sequence analysis of the clones indicated that the size differences are due to the existence of testis-specific exons and alternative RNA splicing (Zhang *et al.*, 1995b). Regarding the role (s) of the LH-like peptides in rat testis, any direct evidence has not been reported yet. This locally produced hormone seems to be functional and be involved in the modulation of spermiogenesis since the transcripts for $LH_β$ and immunoreactive LH molecules are predominantly detected in differentiating spermatids (Zhang *et al.*, 1995a).

The present study was performed to analyze the expression of LH gene in the rat ovary, a female conterpart of testis. Expression of $LH_β$ and $C_α$ gene in the rat ovary was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR). To assess the production of immunoreactive LH-like molecules in rat ovary, radioimmunoassay (RIA) was performed using antibody which can recognize the rat $LH_β$ polypeptide.

MATERIALS AND METHODS

1. Animals and collection of tissue samples

Immature Sprague-Dawley rats (day 25) were ip injected with 15 IU pregnant mare's serum gonadotropin (PMSG; Sigma) or 0.2 ml saline. Rats were sacrificed at different time intervals such as 24, 48 and 72 h after injection, respectively. After decapitation, the morphologies of ovaries and uteri were investigated briefly to ensure the administration of PMSG induced sexual maturation properly, then ovaries and pituitaries were removed and stored at -70℃ until use. Granulosa cells were obtained from ovaries by needle puncture (Lee *et al.*, 1994b). Serum was collected from trunk blood and stored at -20℃ prior to LH radioimmunoassay.

2. LH RIA

Tissues and cell extracts were homogenized in ice-cold 1 M acetic acid. The homogenates were centrifuged at 10,000 g for 10 minutes, and the supernatants were neutralized with 2 N NaOH and used for assay. Tissue contents and serum level of LH were measured by a double antibody RIA method (Lee *et al.*, 1994a). The reference standard (rLH-RD-2) and anti-LH antiserum (anti-rLH-S-6; 1:1,000,000 final dilution) were donated from NIDDK pituitary hormone program (NIH, USA) and the anti-rabbit gamma globulin (ARGG) was purchased from Sigma. The detection limit for the LH assay was 20 pg/tube and the intra-, inter-assay precisions were 7.2 and 9.4%, respectively.

3. RNA Extraction

The total RNA was extracted from the tissues by using TriZol solution (Gibco-BRL, USA), a mixture based on the acid guanidium thiocyanate-phenol-chloroform RNA extraction method (Chomzyncski & Sacchi, 1987). Tissues (100 mg/tube) were homogenized with 1 ml of TriZol solution, subsequently 0.2 ml of chloroform-isoamyl alcohol (49:1) was added to the homogenates with mixing vigorously by hand. Samples were centrifuged at 10,000 g for 20 min at 4℃. After spin, the upper part (aqueous phase) was collected, mixed with 1 ml of isopropanol and placed at -20℃ for 1 h. Sedimentation (at 10,000 g) for 20 min at 4℃ was performed and the resulting pellets were washed in 75% ethanol and dissolved in DEPC-

Table 1. Sequences and positions of primers used in RT-PCR

Set	Name	Sequences	Size (bp)
LH_β-I	P1 (5'-primer) P2 (3'-primer)	GTGCCGGCCTGTCAACGCAAC CAGCTCATTGGTTGAGTCCTG	306
LH_β-II	P3 (5'-primer) P4 (3'-primer)	TGGAGCTCACTGACCACCATC GTACACTGTAGATGCCTGGCA	482
C_α	P5 (5'-primer) P6 (3'-primer)	GCTACAGAAGATATGCGGCTG CGACACTCAGTGCCATCGCAG	418
*	dT	ATAGAATTC-dT_{25}	for RT

Sequneces are all 5' to 3' direction. All oligos were purchased from Korea Bioneer. Primer designs were carried out based on the reported cDNA sequences of rat LH_β and C_α (Godine *et al.*, 1982; Jameson *et al.*, 1984; Zhang *et al.*, 1995b).

treated water. RNA samples were quantitated by UV spectrophotometry at 260 nm and stored at -70℃ until further reaction.

4. RT-PCR

First strand cDNA was synthesized from the total RNA (1 μg) using SuperScript RNase H^- reverse transcriptase (GIBCO-BRL) and oligo $d(T)_{25}$ primer. PCR reactions were performed in a thermal cycler (PTC-150, MJ Research). Generally, the reactions were performed in a total volume of 50 μl containing 5 μl of dNTP-premixed 10 x buffer, 100 pM of each primer, 20~50 ng of template DNA, and 0.25 unit of Taq DNA polymerase (Takara). The final reaction mixture was overlaid with 100 μl of mineral oil (Sigma) to prevent evaporation. Primers and their sequences used in this study are shown in Table 1. Thirty-five to fourty cycles were usually run, and each cycle consists of 1 min at 94℃ (denaturation) 1 min at 52℃ (annealing) and 1 min at 72℃ (extension). In each PCR reaction, a negative control sample (RT mixture without RNA) was used. The amplified cDNA fragments were subjected to 2.0% agarose gel electrophoresis and stained with ethidium bromide (EtBr) for visualizing. To confirm that the cDNAs for LH subunits were specifically amplified, the nucleotide sequence of the PCR products was determined partially by using PCR sequencing kit (Korea Bioneer).

5. Statistics

Student's *t*-test was used for analysis of the differences in tissue contents and serum levels of LH between control and PMSG-injection groups.

RESULTS

1. RT-PCR of the transcripts for rat LH subunits

RT-PCR analysis using the primers for amplification of rat LH_β cDNA yielded a fragment of 306 bp in ovary samples derived from immature rats (Fig. 1A, lane IV). A cDNA fragment of identical size was obtained in pituitary and testis samples which were used as positive controls (Fig. 1A, lanes II and III), while no PCR product was produced in negative control (Fig. 1A, lane IV). To examine whether the rat ovarian LH_β transcripts contain the recently identified testis-specific exons (Zhang *et al.*, 1995b), the second set of primers corresponding to the major testicular LH_β exon 1 of 2.4 kb transcript was used in the next PCR reaction. A 428 bp DNA fragment was obtained from ovarian tissue (Fig. 1B, lane IV), and from testis used as positive control tissue (Fig. 1B, lane III). Negative control reactions,

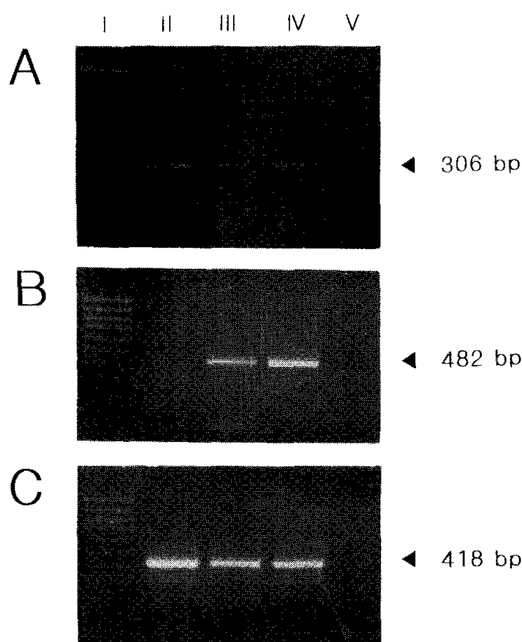

Fig. 1. Detection of the transcripts for rat LH subunits in the rat ovaries by RT-PCR. PCR and electrophoresis were carried out as described in Materials and Methods. **A**; Amplification of cDNA fragments coding the LH_β regions commonly found in the rat pituitary and testis. **B**; A set of primers corresponding the exon 1 of the rat testicular LH_β was used in this PCR. **C**; Amplification of cDNA fragments from the C_α transcripts in pituitary, testis and ovary. Lanes I, 100 bp DNA size marker; II, pituitary; III, testis; IV, ovary; and V, negative control.

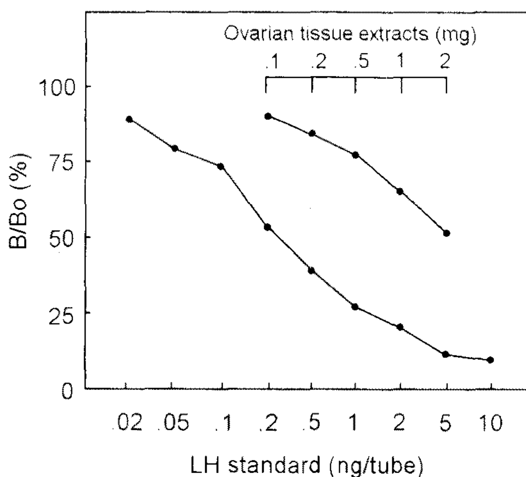

Fig. 2. LH RIA parallelism with increasing amounts of LH standards and the ovarian tissue extracts, indicating the presence of immunoreactive LH-like molecules in the rat ovary. In this experiment, ovaries from immature rats (Day 25) were used.

the pituitary samples and RNA-free RT mixture, did not yield any PCR product (Fig. 1B, lanes II and V).

The amplification of cDNA fragments from the C_α transcripts in pituitary, testis and ovary were shown in Fig. 1C. Partial sequence analysis of the PCR products confirmed that all the PCR reactions were specifically occurred.

2. Competition curves with increasing amounts of LH standard and tissue extracts

Significant amount of LH-like molecules were detected in crude ovarian extracts, and the competition curves with increasing amount of tissue extracts were parallel with those of standard peptide (rLH-RD-2), indicating that the ovarian immunoreactive LH-like material is similar to authentic pituitary LH molecule (Fig. 2). The LH contents in ovarian granulosa cells from both immature and PMSG-injectesd rats were only traceable, the majority of LH molecule might be present in theca-interstitial cells (unpublished data).

3. Effect of PMSG on changes in serum LH levels and ovarian LH contents

The administration of PMSG to immature rats resulted in the stimulation of follicular maturation and endogenous preovulatory LH surge (Bahr & Ben-Jonathan, 1981). As expected, serum LH level after 48 h of PMSG injection significantly increased 5-fold when compared to those of control and PMSG 24 h group. Following then, the LH level declined at PMSG 72 h group, but the level was still higher than that of control and PMSG 24 h group (Fig. 3A). In contrast, single injection of PMSG resulted in a sharp decrease of the ovarian LH content at 24 h, and the lowered level was not recovered at 48 h and 72 h post-injection (Fig. 3B).

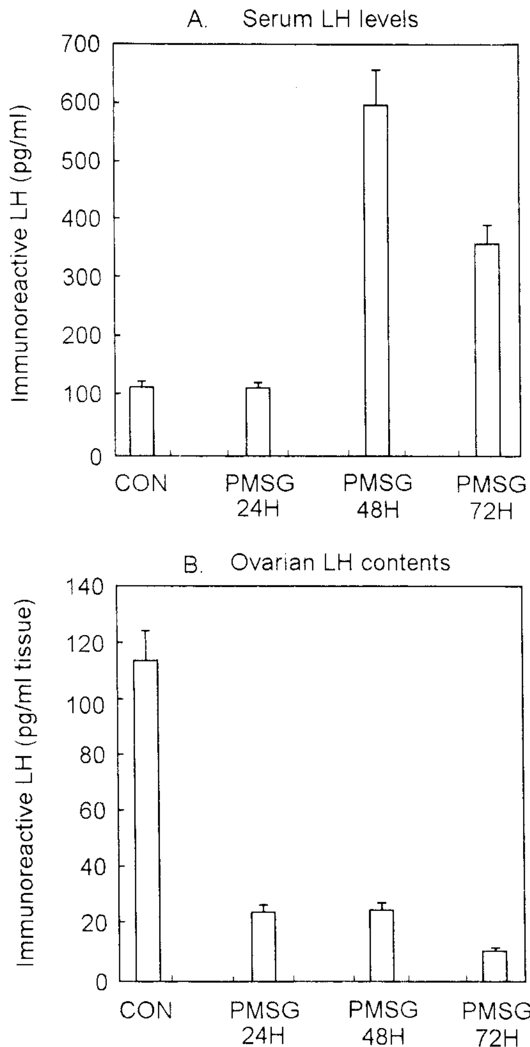

Fig. 3. Differential effect of PMSG on changes in serum LH levels and the ovarian LH contents. CON; sera or tissue extracts from immature rats (day 25). Bar represents the mean level ($\pm$S.E.) of repeated experiments (n=5 to 8).

DISCUSSION

The present results are first to report that genes for the LH subunits are expressed in the rat ovary. This demonstration is based on two evidence; (1) the generation of identical PCR products from two known sources of LH, pituitary and testis, as well as from ovary, and (2) detection of immunoreactive LH-like molecules from ovary by specific LH RIA. The present study also provide evidence that the level of ovarian LH can be regulated physiologically by FSH.

From early 80's, cDNAs and genomic genes of the gonadotropin subunits have been isolated and characterized in several species (Albanese *et al.*, 1996). In eah species, a single gene encodes the C_α and shows considerable size variation (8~16.5 kb). The C_α mRNAs range in size from 0.73~0.8 kb in mammalian species so far analyzed, and the translated product has a signal peptide 24 amino acids in length and the mature peptide has 92-96 amino acids in all species (Ryan *et al.*, 1988). Gene encoding the rat pituitary LH_β is relatively small, with total length of 1.5 kb composed of three exons and two introns, and the transcript is 0.8 kb in size (Jameson *et al.*, 1984).

Until fairly recently, the anterior pituitary gland was the only known source of LH. In fact, the finding of testicular LH expression was quite accidental, since the testis was originally considered for the negative control (Zhang *et al.*, 1995a). The rat C_α mRNA had a size of 0.8 kb in the pituitary and testis, while the major LH_β transcript was 0.8 and 2.7 kb in the rat pituitary and testis, respectively. Subsequently, three transcriptional variants for the rat testicular LH_β with length of 3.2, 2.4, and 0.86 kb were identified and their sequence analysis indicated that the size differenence of these variants and the pituiatry LH_β was due to alternative splicing and differences at the 5' ends of transcripts (Zhang *et al.*, 1995b). RT-PCR amplification in the present study demonstrated that the ovarian LH_β tranascripts contain at least two parts of the published cDNA structure; the pituitary exons 1, 2 and 3 including the translated region which were detected in the pituitary and testis, as well as the part of testicular exons 1 and 2 found in the major LH_β transcripts (2.7 kb) in the rat testis. RT-PCR for amplification of the C_α transcripts shown the identical cDNA product from the

positive control tissues (pituitary and testis) and ovary, indicating that the LH subunits peptides might be produced in the rat ovary. Actually, immunoreactive LH-like molecules were detected in ovary using RIA, and the molecules seem to be physiological substance because the ovarian contents of LH-like were significantly altered by single injection of PMSG.

Mammalian ovary and testis show extreme morphological differeneces due to the sexual dimorphism. In many aspects, however, these organs share common functional natures, such as germ cells production, synthesis and secretion of gonadal factors, and regulation of their functions by pituitary gonadotropins (Griffin, 1996; Ojeda, 1996). In the few years studies provided clear evidence for the production of LH or LH-like molecule in rat testis, so it was plausible to hypothesize that LH might be expressed in rat ovary. In fact, the present study demonstrated that genes for LH subunits are expressed in the rat ovary, and the presence of "gonadal LH" and indicated that the LH_{β} in both ovary and testis might be expressed in gonad-specific manner with alternative splicing mechanism (s) and unique transcription start sites. A similar phenomenon has also been reported with some hormone genes, classically assorted as "non-gonadal", are expressed in rat gonads, *e.g.* growth hormone releasing hormone (GHRH) and pituitary adenylate cyclase activating polypeptide (Bagnato *et al.*, 1992; Srivastava *et al.*, 1995; Hurley *et al.*, 1995; Gräs *et al.*, 1996).

Most of hormones are acting on gonad through an endocrine control mechanism. The major ovarian function is primarily regulated by pituitary gonadotropins that transverse the general circulation to reach gonad. However, there is ample evidence that intraovarian factors are indispensible for fine tuning in the regulation of highly complex but harmonized events in ovary (Richards, 1994). Together, gonadotropins, steroids and locally produced peptidergic factors consist a biological network that modulates the follicular maturation, ovulation, corpus luteum formation and luteolysis (Adashi & Rohan, 1992). In this context, it is intriguing that LH may exert its role on the control of both male and female reproduction through dual pathways. One is an endocrine mechanism for primary action of pituitary LH, the other is an auto- and/or paracrine mechanism for fine tuning action of the locally produced LH. However, the physiological significances such as intraovarian roles, interactions between other trophic factors, and tissue-specific regulation mechanism of the rat ovarian LH remain to be elucidated.

SUMMARY

The present study was performed to analyze the expression of LH genes in the rat ovary. Expression of LH subunit genes in the rat ovary was demonstrated by amplification of ovarian RNA by RT-PCR. The ovarian LH_{β} transcripts contained at least two parts of the published cDNA structure, the pituitary exons 1, 2 and 3 and the part of testicular exon 1 in the major trancripts form in rat testis. Using RIA, significant amount of LH-like molecules were detected in crude ovarian extracts, and the competition curves with increasing amount of tissue extracts were parallel with those of standard peptide, indicating that the ovarian immunoreactive LH-like material is similar to authentic pituitary LH molecule. The administration of PMSG to immature rats resulted in a sharp decrease of the ovarian LH contents after 24 h post-injection. In conclusion, these findings demonstrate that genes for LH subunits are expressed in the rat ovary, and suggest that LH can play a central role in regulation of female reproduction with both endocrine (by pituitary LH) and auto- and/or para-crine (by ovarian LH) manner.

REFERENCES

Adashi EY, Rohan RM: Intraovarian regulation: peptidergic signaling systems. *Trends Endocrinol Metab* 1992, 3, 243-248.

Albanese C, Colin IM, Crowley WF, Ito M, Oestell RG, Weiss J, Jameson JL: The gonadotropin genes: evolution of distinct mechanisms for hormonal control. *Recent Prog Horm Res* 1996, 51, 23-58.

Bagnato A, Moretti C, Ohnishi J, Frajese G, Catt KJ: Expression of growth hormone releasing-hormone gene and its peptide product in the rat ovary. *Endocrinology* 1992, 130, 1097-1102.

Bahr J, Ben-Jonathan N: Preovulatory depletion of ovarian catecholamines in the rat. *Endocrinology* 1981, 108, 1815-1820.

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162, 156-159.

Fiddes JC, Goodman HM: The gene encoding the common alpha subunit of the four glycoprotein hormones. *J Mol Appl Genetics* 1981, 1, 3-18.

Godine JE, Chin WW, Habener JF: Alpha subunit of rat pituitary glycoprotein hormones: primary structure of the precursor determined from the nucleotide sequence of cloned cDNAs. *J Biol Chem* 1982, 257, 8368-8371.

Gräs S, Hannibal J, Georg B, Fahrenkrug J: Transient periovulatory expression of pituitary adenylate cyclase-activating polypeptide in the rat ovarian cells. *Endocrinology* 1996, 137, 4779-4785.

Griffin J: Male reproductive function. In: Griffin J, Ojeda S, eds. Textbook of Endocrine Physiology. New York: Oxford University Press, 1996, 210-222.

Hurley JD, Gardiner JV, Jones PM, Bloom SR: Cloning and molecular characterization of complementary deoxyribonucleic acid corresponding to a novel form of pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 1995, 136, 550-557.

Lee SH, Song ES, Yu SK, Kim C, Lee DK, Choi WS, Kim K: Temporal changes in ovarian gonadotropin-releasing hormone mRNA levels by gonadotropins in the rat. *Mol & Cells* 1994a, 4, 39-44.

Lee SH, Choi WS, Kim C, Kang HM, Geum DH, Park HS, Park YM, Lee CC, Kim K: A local regulation of GnRH release and gene expression in rat granulosa cells by endogenous opioid and GnRH analogues. *Mol & Cells* 1994b, 4, 473-479.

Ojeda S: Female reproductive function. In: Griffin J, Ojeda S, eds. Textbook of Endocrine Physiology. New York: Oxford University Press, 1996, 164-200.

Pierce JG, Parsons TF: Glycoprotein hormones: structure and function. *Ann Rev Biochem* 1981, 50, 465-495.

Richards JS: Hormonal control of gene expression in the ovary. *Endocr Rev* 1994, 15, 725-751.

Ryan RJ, Chalesworth MC, McCormick DJ, Milius RP, Keutmann HT: The glycoprotein hormones: recent studies of structure-function relationships. *FASEB J* 1988, 2, 2661-2669.

Srivastava CH, Monts BS, Rothrock JK, Peredo MJ, Pescovitz OR: Presence of spermatogenic-specific promoter in the rat growth hormone releasing-hormone. *Endocrinology* 1995, 136, 1502-1508.

Zhang F, Markkula M, Toppari J, Huhtaniemi I: Novel expression of luteinizing hormone subunit genes in the rat testis. *Endocrinology* 1995a, 136, 2904-2912.

Zhang F, Rannikko A, Huhtaniemi I: Isolation and characterization of testis-specific cDNAs for luteinizing hormone β-subunit in the rat. *Biochem Biophys Res Commun* 1995b, 210, 858-865.