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Cloning and Expression of *FSHb* Gene and the Effect of FSHβ on the mRNA Levels of *FSHR* in the Local Chicken*

L. H. Zhao, J. L. Chen[1], H. Xu, J. W. Liu and R. F. Xu**

College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China

ABSTRACT : Follicle-stimulating hormone (FSH) is a pituitary glycoprotein hormone that is encoded by separate alpha- and beta-subunit genes. It plays a key role in stimulating and regulating ovarian follicular development and egg production in chicken. FSH signal transduction is mediated by the FSH receptor (FSHR) that exclusively interacts with the beta-subunit of FSH, but characterization of prokaryotic expression of the *FSHb* gene and its effect on the expression of the *FSHR* gene in local chickens have received very little attention. In the current study, the cDNA fragment of the *FSHb* gene from Dagu chicken was amplified using reverse transcription polymerase chain reaction (RT-PCR), and inserted into the pET-28a (+) vector to construct the pET-28a-FSHb plasmid. After expression of the plasmid in *E. coli* BL21 (DE3) under inducing conditions, the recombination protein, FSHβ subunit, was purified and injected into the experimental hens and the effect on the mRNA expression levels of the *FSHR* gene was investigated. Sequence comparison showed that the coding region of the *FSHb* gene in the local chicken shared 99%-100% homology to published nucleotides in chickens; only one synonymous nucleotide substitution was detected in the region. The encoded amino acids were completely identical with the reported sequence, which confirmed that the sequences of the chicken *FSHb* gene and the peptides of the FSHβ subunit are highly conserved. This may be due to the critical role of the normal function of the *FSHb* gene in hormonal specificity and regulation of reproduction. The results of gene expression revealed that a recombinant protein with a molecular weight of about 19 kDa was efficiently expressed and it was identified by Western blotting analysis. After administration of the purified FSHβ protein, significantly higher expression levels were demonstrated in uterus, ovary and oviduct samples ($p<0.05$). These observations suggested that the expressed FSHβ protein possesses biological activity, and has a potential role in regulation of reproductive physiology in chickens. (**Key Words :** Chicken, *FSHb* Gene, Expression, FSH-receptor)

INTRODUCTION

Follicle-stimulating hormone (FSH) is a member of the glycoprotein hormones synthesized and secreted by gonadotropes in the anterior pituitary gland and is comprised of two dissimilar subunits, alpha and beta, encoded by separate genes (Jameson et al., 1988; Gharib et al., 1989). Within a species, FSH shares the identical α subunit to luteinizing hormone (LH) and thyroid-stimulating hormone (TSH), while it has its own β subunit that is different and determines both hormonal and species specificity (Pierce and Parsons, 1981). It acts in an endocrine manner and play an essential role in the reproductive system, including steroidogenesis, folliculogenesis and follicular maturation (Choi et al., 2005), and in regulating gonadal endocrine function (Moyle and Campbell, 1996). The latest studies have revealed that FSH exists in two glycoforms: diglycosylated FSH (DiGlycFSH) and tetraglycosylated FSH (TetGlycFSH). It also specifically functions to stimulate estrogen synthesis and serves as a selection factor for dominant follicles, which are essential to maintain fertility (Bousfield et al., 2007; Tran et al., 2008). In humans, DiGlycFSH involves expression of recombinant hFSHβ, separation, purification from soluble and insoluble fractions, folding, and re-association with human chorionic gonadotropin (hCGα) (Tran et al., 2008).

The primary amino acid sequence of the FSHβ subunit

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** Corresponding Author: Ri Fu Xu. Tel: +86-431-84531124, Fax: +86-431-84533425, E-mail: rufusxu@yahoo.com.cn

[1] Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100094, China.

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was earlier determined from human pituitary glands, and consists of 118 amino acid residues with a predominant proportion of molecules having 108 residues due to microheterogeneity at the NH_2 and COOH termini (Saxena and Rathnam, 1976). The *FSHb* gene encoding FSHβ has been isolated in the human, that encoded a 18 amino acid signal sequence and a 111 amino acid apoprotein (Watkins et al., 1987; Jameson et al., 1988); in the rat, that encoded a 20 amino acid signal peptide followed by a mature protein of 110 amino acid residues (Maurer, 1987; Gharib et al., 1989), in the porcine (Kato, 1988), in the bovine (Esch et al., 1986; Maurer and Beck, 1986), in Chinese hamster (Keene et al., 1989), in the ovine (Guzman et al., 1991), and the equine (Saneyoshi et al., 2001). In the chicken, the FSHβ precursor molecule consisted of 131 amino acids with a signal peptide of 20 amino acids followed by a mature protein of 111 amino acids that was encoded by *FSHb* cDNA, which was composed of 2,457 bp nucleotides, including 44 bp nucleotides of the 5`-untranslated region (UTR), 396 bp of the open reading frame, and a long 3`-UTR of 2,001 bp nucleotides followed by a poly$(A)_{(16)}$ tail (Shen and Yu, 2002). The effects of FSH on ovarian follicular development and growth have given rise to the hypothesis that FSH functions to enhance laying performance, or egg production, in the chicken, but the beta subunits of FSH vary in different species. It confers its specific biologic action and is responsible for interaction with the FSH receptor (Simoni et al., 1997).

The FSH receptor (FSHR) is a member of the super-family of G-protein-coupled receptors; it was reported to be expressed exclusively on granulosa cells of the ovary in females and on sertoli cells of the testis in males, which mediate FSH signal transduction through the cAMP pathway (Griswold et al., 1995; Simoni et al., 1997). FSH indirectly influences spermatogenesis and oogenesis by exerting influence on the corresponding somatic cells (Griswold et al., 1995). Both granulosa cells and immature sertoli cells are capable of synthesizing estrogen from an exogenous source of androgens (Fritz, 1982). Regulation of the expression of FSHR and its connection with specific signal transduction pathways are key points in regulating folliculogenesis (Findlay and Drummond, 1999). With respect to regulation of the FSHR gene expression and effects on the expression level of FSHR mRNA, extensive studies have been undertaken on the granulosa cells of the ovary and sertoli cells of the testis in the rat (Camp et al., 1991; Heckert and Griswold, 1991), mouse (Yaron et al., 1998), sheep (Tisdall et al., 1995), bovine (Xu et al., 1995), human (Oktary et al., 1997; Findlay and Drummond, 1999), and chicken (Yamamura et al., 2001; Woods and Johnson, 2005). Of these, most experiments were focused on response of the sampled tissues or cells to FSH and its roles in folliculogenesis and spermatogenesis. While in the tissue distribution and expression levels of *FSHR* mRNA, and response to FSHβ subunit, very little information is available in Chinese indigenous chickens. As for the preparation of recombinant FSHβ subunit and identification of its biological activity, work has been reported rarely in chickens. The objective of this study was to investigate the expression characteristics of *FSHR* mRNA in the different tissues sampled, to obtain the purified recombinant FSHβ subunit, and to evaluate the effect of muscular injection of a dose of the FSHβ on the mRNA expression levels of FSHR in Chinese indigenous layers. The results will hopefully establish a foundation for study of the physiological activity and characteristics of chicken FSHβ subunit in regulation of egg performance.

MATERIALS AND METHODS

Animals and RNA isolation

In this study, pituitary gland, uterus, ovary, oviduct, pancreas, glandular stomach and kidney samples were collected at 18 weeks of age from Dagu chicken, a Chinese indigenous breed, and the tissues were snap-frozen in liquid nitrogen within 5 min of sacrifice. Total RNA was extracted using TRIzol Reagent according to the manufacturer's protocols (Gibco BRL, Grand Island, NY), the concentration of total RNA was quantified by measuring its absorbance at 260 nm, and the purity was detected from the ratio of absorbance at 260/280 nm.

RT-PCR analysis

To identify the tissue specificity of chicken *FSHb* mRNA expression, RT-PCR analysis of *FSHb* and constitutively expressed β-actin was carried out as described previously (Zheng et al., 2006; Wu et al., 2008). The cDNA was synthesized by reverse transcription using 1 μg total RNA according to the instructions of the reagent kit (Reverse Transcriptase kit, Takara). The PCR reaction was performed in a volume of 20 μl consisting of 1 μl of RT-PCR product, 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM each primer and 1 U Taq DNA polymerase (MBI Fermentas). The PCR conditions were 95°C for 5 min followed by 30 cycles of denaturing at 95°C for 40 s, annealing at 59°C for 40 s, extension at 72°C for 40 s, and final extension at 72°C for 4 min, and finally kept at 4°C. The amplification was performed on a PTC-100™ programmable thermal controller (MJ Research, Inc.). The β-actin gene was used as an internal control. The following sets of oligonucleotide primers were used: for *FSHb*, 5'-GCCAACGAGATACAACCTACTTCC-3' and 5'-AGGCAT GAACTGGATAGACTTCGTGC-3'; for β-actin, 5'-ATCA GCAAGCAGGAATACGA-3' and 5'-CGGCAGCAACAG AAGTGGA-3'. The primers were designed according to the

published sequences of chicken *FSHb* (Accession No. NM_204257) and β-actin (M26111) mRNA in the NCBI GenBank database. The reaction products (4 μl) were visualized and photographed following electrophoresis on 2% agarose gels stained with 0.25 μg/ml ethidium bromide, and the expected product was extracted and verified by direct DNA sequencing. To verify the specificity of the bands shown, a negative control was performed using an aliquot of a cDNA synthesis reaction where no reverse transcriptase enzyme was added.

Cloning of the encoding region of chicken *FSHb* gene

The total volume of the PCR reaction system was 50 μl, containing 1 μl of the above synthesized cDNA template, 0.25 μM of each primer, and 1 U of Taq DNA polymerase, to amplify 396 bp of the full encoding region cDNA of the chicken *FSHb* gene. The conditions for PCR were pre-amplification denaturation at 94°C for 5 min followed by 30 thermal cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 4 min. The primers were also designed on the basis of the aforementioned *FSHb* mRNA sequence (NM_204257) and optimized for *E. coli* codon usage. *EcoR*I and *Sal*I restriction enzyme sites were incorporated in the primers: the forward primer 5'-TCG**GAATTC**ATGAAGACACTTAACTGT-3', and the reverse primer 5'-AG**GTCGAC**TCATTGATTGCTTCCA TTG-3', corresponding to the 45-440 nt of follicle stimulating hormone, beta polypeptide (*FSHb*) mRNA. The PCR products were isolated with 1.5% agarose gel electrophoresis and purified with a DNA purification kit (MiniBEST DNA Purification Kit, Takara). The amplified fragment was inserted into pGEM-T easy vector using T4 DNA ligase (Promaga); recombinant DNAs were introduced by heat shock into *E. coli* DH5α for propagation. Positive clones with correct insertions were identified using PCR and restriction analysis of plasmids carrying the recombinant cDNAs. The recombinant plasmids were then subjected to DNA sequence analysis, and selected for construction of expression vector with the obtained *FSHb* fragment.

Sequence alignments, translations and comparisons were carried out using MEGA program Version2.1 (Kumar et al., 2001), and the deduced amino acids of the FSHβ subunit corresponding to the 21 sequences of *FSHb* mRNA from 13 species (Table 1) were used to perform sequence comparison.

Expression of chicken *FSHb* gene in *Escherichia coli* BL21 (DE3)

The chicken *FSHb* cDNA was cloned into *EcoR*I and *Sal*I restriction sites of the pET-28a (+) vector to construct the pET-28a-FSHb plasmid. The recombinant plasmid was confirmed by restriction analysis and transformed into *E. coli* BL21 (DE3) cells. The positive clone was selected for incubation overnight at 37°C with shaking in LB medium (10 ml) containing 34 mg/L kanamycin. This culture was used to inoculate the pre-warmed new LB medium containing 34 mg/L kanamycin with a 1:100 dilution at 37°C with shaking. When the culture had grown to an OD_{600} of 0.6-0.8, the expression of recombinant *FSHb* was induced by adding isopropyl-β-D-thiogalactopy-ranoside (IPTG) at 0.1 M final concentration, and incubating for 1, 2, 3 and 4 h at 37°C. The cultures were collected and analyzed by 12% SDS-PAGE as described by Laemmli (Laemmli, 1970). The fusion protein was identified using a commercially available monoclonal anti-FSHβ protein (Santa Cruz Biotechnology) by Western blotting assay (Barnes et al., 2002; Lu et al., 2007).

Table 1. Homologies of the coding regions of *FSHb* genes and the deduced amino acid sequences of FSHβ subunits used in this study

Species	GenBank Accession No.	Sequence homology (%) cDNA	amino acid
Chicken	NM_204257	100	100
	AB077362	100	100
	AF467082	99	100
Broiler chicken	BI392995	100	100
Japanese quail	AB086952	97	98
Domestic duck	DQ232890	95	99
Chinese goose	EU563910	95	97
Crested ibis	AB089502	90	96
Norway rat	BC168724	72	64
	NM_001007597	72	64
House mouse	BC061159	72	65
Human	NM_000510	73	66
	BC113490	73	66
Hamster	AB252645	74	66
Golden hamster	AB241062	73	65
Chinese hamster	AB248599	73	67
Porcine	NM_213875	74	69
Bovine	NM_174060	73	65
Ovine	NM_001009798	72	66
Equine	AB029157	72	65

Recombination protein extraction and purification

The following purification was performed using cells derived from one liter of IPTG-induced bacterial culture. The fusion protein was purified from *E. coli* BL21 by the methods described previously (Dai et al., 2007). In brief, the cultured cells were collected by centrifugation and re-suspended. The samples was lysed by ultrasonication. The lysate was centrifuged at 12,000 rpm for 30 min and 2% sodium deoxycholate was added to the inclusion body; the

body was collected by centrifugation and re-suspended. The sample was centrifuged at 12,000 rpm for 30 min; all steps were performed at 4°C and then 20% PEG-4000, 50 mM oxidized glutathione, and 100 mM reduced glutathione were added to the supernatant to final concentrations of 0.2%, 1 mM and 2 mM, respectively. The expressed protein was then concentrated by PEG-20000.

Effect of FSHβ on the expression of *FSHR* mRNA in hens

Ten healthy Dagu hens at 23 weeks of age were divided randomly into two groups. They were housed in individual cages and raised under the same conditions of management and nutrition on the farm of the Jilin Agricultural University. One group was administered a pectoral muscle injection of 10 μg/ml of purified FSHβ protein dissolved in 0.9% sodium chloride. The first administration of FSH was equal to a dose of 5 μg, the second of 15 μg per bird. At the same time, the other group was used as the control and injected with an equal amount of 0.9% sodium chloride. After two successive injections, given 3-4 h apart based on the half-life of FSH (Laster, 1972), tissue samples of pituitary gland, uterus, ovary, oviduct, pancreas, glandular stomach and kidney were collected from the sacrificed hens. Before treatment, tissues from another five non-treated Dagu chickens at 23 weeks of age were collected to reveal expression of the *FSHR* gene. To measure mRNA expression level of the chicken *FSHR* gene in the tissues, semi-quantitative RT-PCR analysis of *FSHR* and β-actin was performed as described above. The primers were designed on the basis of published chicken *FSHR* (GenBank accession No. NM_205079) mRNA sequences: 5'- TAAGAGCGAGGTCTACATACA -3' and 5'-GTGGTGTTCCCAGTGATAG -3', corresponding to the 786-1,200 nt of follicle stimulating hormone receptor (FSHR) mRNA. The reaction was carried out for 30 cycles, each comprising denaturing at 95°C for 40 s, annealing at 60°C for 40 s, extension at 72°C for 40 s, and final extension at 72°C for 4 min.

Statistical analysis

All quantified results are expressed as means±SE. The results were assessed using the Kruskall-Wallis non-parametric test by the software package SPSS11.0 (Conover, 1999; Lin et al., 2002). Statistical significance was indicated with a p value <0.05.

RESULTS

The tissue specificity of *FSHb* mRNA expression analyzed by RT-PCR

As shown in Figure 1, during chicken ovary development, higher *FSHb* mRNA expression levels were observed in the pituitary gland tissues of Dagu chickens sampled at 18 weeks of age. However, *FSHb* mRNA expression in other tissues sampled, namely uterus, ovary, oviduct, pancreas, glandular stomach and kidney, was not observed when an equal amount of total RNA was used under the same RT-PCR reaction conditions. In combination with the results previously reported by Shen and Yu (2002) that RNAs from other tissues (brain, heart, thyroid, liver, testis, and adipose) did not produce this cDNA fragment under the same RT-PCR conditions, this observation indicated that the *FSHb* mRNA was specifically expressed in the pituitary gland tissue of the chicken.

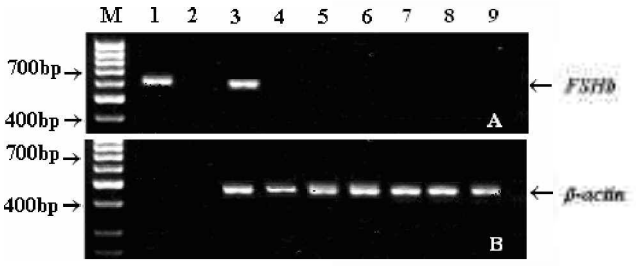

Figure 1. The mRNA expression of chicken *FSHb* in the tissues sampled. Lane1: presenting the controls: group +, using total RNA from chicken pituitary gland sample without primers for β-actin; Lane 2: group -, performed using an aliquot of a cDNA synthesis reaction where no reverse transcriptase enzyme was added; Lane 3: pituitary gland, lane 4: uterus, lane 5: ovary, lane 6: oviduct, lane 7: pancreas, lane 8: glandular stomach, and lane 9: kidney samples. A: 647 bp band for *FSHb*, B: 461 bp band for β-actin.

Sequence analysis and expression of chicken *FSHb* gene

A cDNA fragment, which comprised an open reading frame of 396 bp encoding 131 amino acid residues (with the exception of a stop codon), was amplified from first strand cDNA prepared from total RNA of chicken pituitary gland. The cDNA sequence carried in the recombinant plasmid with correct insertion was identified by PCR and restriction analysis (Figure 2). Sequence of the coding region with its 3`-untranslated region of the chicken *FSHb* gene was submitted to the NCBI GenBank and assigned the accession number of GQ856365. Sequence analysis revealed that the *FSHb* gene of poultry had a length of 396 bp nucleotides encoding a peptide with 131 amino acid residues; rat and mouse had a 393 bp the coding region of *FSHb* encoding 130 amino acids; but in each of human, hamster, porcine, bovine, ovine and equine had the same length of 390 bp nucleotides encoding a peptide of 129 amino acids. By alignment, the inserted nucleotide fragment shared 99% and 100% identities to reported chicken *FSHb* sequences (AF467082 and NM_204257); only one synonymous nucleotide substitution, $A^{345}\rightarrow G$, was detected in the region. The deduced amino acid sequence from the newly isolated

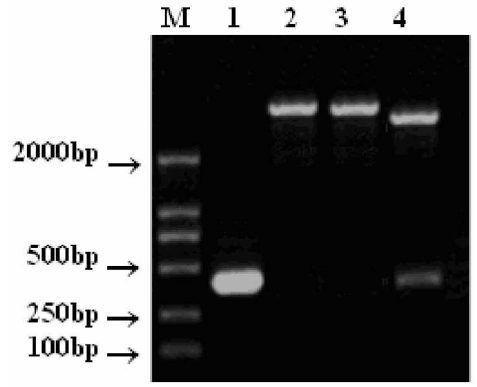

Figure 2. Identification of the recombinant plasmid pGEM-cFSHb using PCR and digestion of endonucleases of *EcoR*I and *Sal*I. M, DL 2000 marker; Lane1, PCR product of recombinant pGEM-cFSHb; Lane 2, digestion of pGEM-cFSHb with enzyme *EcoR*I; Lane 3, digestion of pGEM-cFSHb with *Sal*I; Lane 4, digestion of pGEM-cFSHb with *EcoR*I and *Sal*I.

fragment shared 100% similarity to those published from the chicken. This result confirmed that the sequences of the chicken *FSHb* gene and the peptides of the FSHβ subunit are highly conserved. Moreover, the sequence homologies of the cloned chicken segment with quail, duck, goose and other animals are listed in Table 1. The comparison of the deduced amino acids of the FSHβ subunit corresponding to the 17 sequences of FSHb mRNA from 13 species is shown in Figure 3. It shows the two asparagine (Asn) at position 5 and 22 of the mature chicken FSHβ subunits for the N-linked glycosylation (Ulloa-Aguirre and Timossi, 1998); and 12 cysteine (Cys) sites for disulphide bonds (Ryan et al., 1987), and the regions forming a 'seatbelt' (Chopineau et al., 2001).

The positive *E. coli* BL21 (DE3) cells harboring pET-28a-FSHb were cultured. The expression of recombinant FSHβ protein is shown in Figure 4. On SDS-PAGE analysis under the induced conditions, the recombinant protein was highly expressed in *E. coli* BL21 after inducing expression by IPTG at 0.1 M final concentration for 3 h and 4 h. A recombinant protein band with molecular weight of about 19 kDa was detected, which corresponded to the estimated molecular weight of the chicken FSHβ subunit of about 14.74 kDa. The expressed FSHβ from *E. coli* BL21 was purified according to the method aforementioned (shown in Figure 4, lane 7).

As shown in Figure 5, the expressed recombinant molecule was identified using a commercially available monoclonal anti-FSHβ protein by Western blotting analysis. The expressed protein was recognized by the monoclonal antiserum. The purified and un-purified recombinant

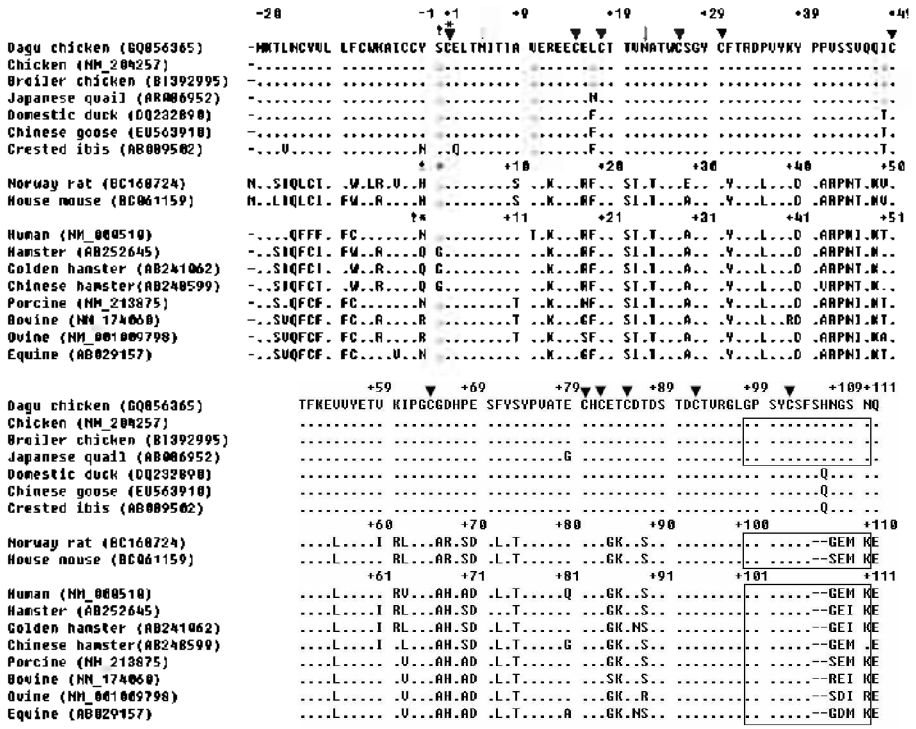

Figure 3. Comparison of the deduced amino acid sequences of FSHβ subunits corresponding to the 17 sequences of FSHb mRNA from 13 species. The asterisk (*) and exclamation mark (!) at top indicate the position of the first amino acid residues of the mature FSHβ subunits and the last residue of the signal peptides, respectively. The arrow (↓) donates the position of N-linked glycosylation, the arrowhead (▼) marks the 12 cysteine (Cys) sites for disulphide bond, the dot (•) shows identity to the peptide sequence of the FSHβ subunit, and the boxes indicate the regions forming a 'seatbelt' (Chopineau et al., 2001).

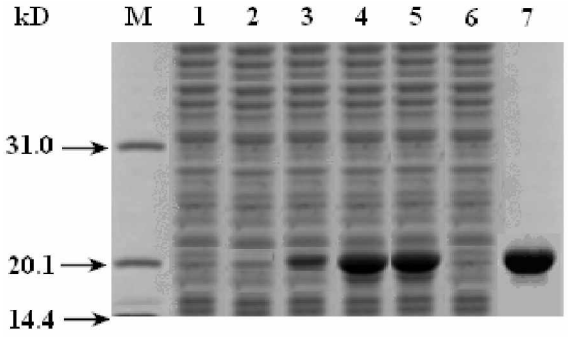

Figure 4. SDS-PAGE analysis of the expression of recombinant chicken *FSHb* gene in *E. coli* BL21 (DE3). M, Protein marker; Lane 1, pET-28a (+)-cFSHb vector without induction in *E. coli* BL21 (DE3); Lane 2-5, pET-28a (+)-cFSHb vector induced in E.coli BL21 (DE3) for1 h, 2 h, 3 h and 4 h, respectively. Lane 6, pET-28a (+) vector induced in *E. coli* BL21 (DE3) for 4 h. Lane 7, purified recombinant chicken FSHβ protein.

proteins are shown in lane 1 and 2, respectively, of Figure 5.

Effect of FSHβ subunit on the expression of *FSHR* mRNA in hen

To understand the effects of the FSHβ subunit on the expression of *FSHR* mRNA in hens, semi-quantitative RT-PCR was developed to characterize expression of the *FSHR* gene in pituitary gland, uterus, ovary, oviduct, pancreas, glandular stomach and kidney tissues. The results showed expression of *FSHR* mRNA in uterus, ovary, oviduct, pancreas and glandular stomach in the three groups; whereas the expression levels of *FSHR* mRNA in the non-gonadal tissues were much lower than in the ovary. The levels of mRNA expression in the control chickens were not significantly different from those detected before treatment by injection of FSHβ protein or 0.9% sodium chloride ($p>0.05$), but significantly higher expression levels were demonstrated in uterus, ovary and oviduct samples of the trial group than in the control ($p<0.05$). No expression was detected in pituitary gland and kidney tissues of the three groups (Figure 6).

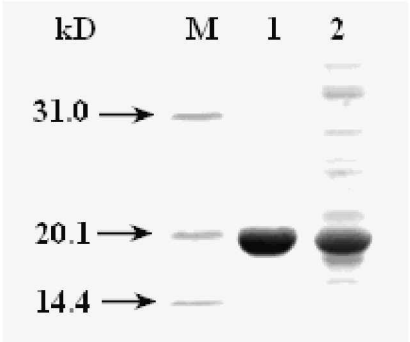

Figure 5. Western blotting analysis for the recombinant chicken FSHβ protein. M, protein marker; Lane 1, purified recombinant protein; Lane 2, un-purified recombinant protein.

DISCUSSION

Chicken FSH plays an essential role in the control of pubertal maturation and reproductive processes. Specifically, it initiates ovarian follicular development and stimulates the maturation of germ cells in hens (Rao et al., 1978; David et al., 2007). Related studies showed that there was a firm positive correlation during laying between chicken FSH levels in plasma and egg laying performance (Yang et al., 2007). It is well accepted that multiple regulatory mechanisms govern the release of FSH. The levels of FSH secretion are mainly regulated by the hypothalamic-pituitary-gonadal axis (HPGA) (Padmanabhan et al., 2002). Extensive studies have been made of the physiological function of FSH and associated regulative factors and stimuli (activin) or inhibins (Lovell et al., 2001), but several questions have still received very little attention.

Considering that FSH is comprised of two dissimilar subunits, α and β, encoded by separate genes, of which the FSHβ subunit is responsible for interaction with the FSHR

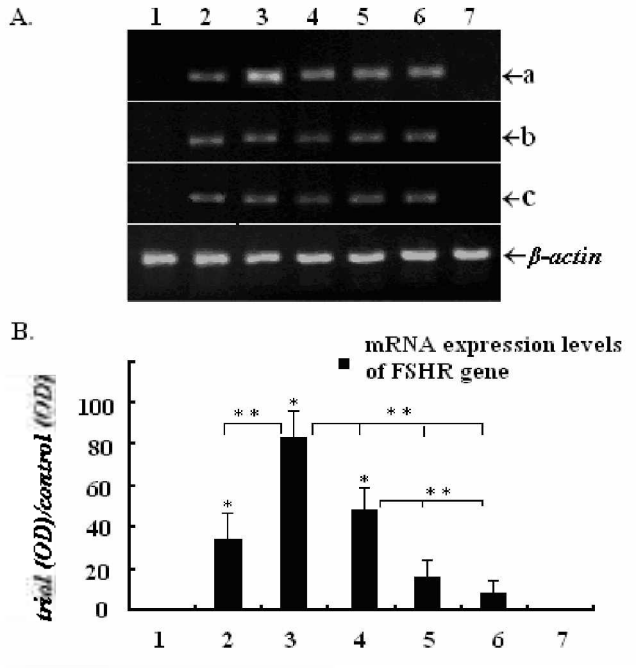

Figure 6. The expression level of *FSHR* mRNA in chicken detected by RT-PCR. A, Electrophoresis of *FSHR* mRNA expression; lane 1: pituitary gland, lane 2: uterus, lane 3: ovary, lane 4: oviduct, lane 5: pancreas, lane 6: glandular stomach and lane 7: kidney. a: trial group; b: before treatment; c: control group. B, Histograms of ratio of expression levels of *FSHR* mRNA in the trial and control groups, columns 1-7 corresponding to lanes 1-7, respectively. The histogram marked with asterisk (*) indicates a significant difference between the trial and control groups in the same tissues at $p<0.05$, and double asterisks (**) denote statistical differences between the various tissues ($p<0.05$).

(Tomas et al., 1990; Simoni et al., 1997), then what effect does a single FSHβ subunit exclusively have on the mRNA expression of the *FSHR* gene? Furthermore, how does the *FSHb* gene contribute to the diversification of ovarian follicular development and egg production in the chicken? In this study, we have firstly isolated the coding region of the *FSHb* gene encoding the FSH beta-subunit from Dagu chickens at 18 weeks of age. The high identities of the coding region sequence of the *FSHb* gene and the deduced amino acid sequence from the Dagu breed to those of chicken (broiler), quail, domestic duck, geese and crested ibis, indicated that the coding region of the chicken *FSHb* gene and the amino acid sequence of the FSHβ subunit were highly conserved. This may be due to the crucial role of the normal function of the *FSHb* gene in the hormonal specificity and regulation of reproduction within a species. However, why do, the various effects of the *FSHb* gene on laying performance of chickens exist? Possible reasons may be explained by the following: i) Polymorphism of the chicken *FSHb* gene mainly comes from its un-translated regions except for its two introns in the genomic sequence. In Dagu chicken, the 3`-untranslated region of *FSHb* mRNA showed 97-98% similarity to each other within the breed sampled (unpublished material). In Taihu and Wenchang chicken, six nucleotide variations were identified in the promoter region of the *FSHb* gene; some of them were significantly correlated with egg production of layers (Hong, 2004; Han, 2006). ii) The activity of the FSHβ subunit determines its specific biologic activity and is responsible for interaction with the FSH receptor (Tomas et al., 1990; Simoni et al., 1997). FSHR is essential for integrating the pituitary FSH signal with gonadal response (Heckert, 2001). iii) Both *FSHb* and *FSHR* genes play important roles in control of the hypothalamic-pituitary-gonadal axis. The rare mutations of the FSHβ subunit were shown to produce distinctive phenotypes in reproductive physiology (Themmen and Huhtaniemi, 2000; Chopineau et al., 2001). iv) Coordination of the FSHβ subunit with other factors, such as the FSH alpha-subunit, luteinizing hormone (LH) and steroidogenic factor 1 (SF-1), can promote the response of the gonads to FSH (Heckert, 2001), correspondingly stimulating dramatically dominant follicular growth, and triggering ovulation of the follicle (Chappel and Howles, 1991).

Sequence comparison demonstrated that the deduced amino acids of the FSHβ subunit are highly conserved, whereas different polymorphism patterns of the rare variations of amino acids in the variable sites appear among the various species. It has been reported that within the sequences of vertebrate FSHβ subunit proteins, the region at the 100-110 (Gly-Pro-Ser-Tyr-Cys-Ser-Phe-Gly-Glu-Met-Lys) in humans has an impact on FSH specificity (Moyle et al., 1994). The 100-110 sequence of the FSHβ subunit surrounds the α-subunit forming a 'seatbelt' which is fastened by the 26-110 disulfide bond (Chopineau et al., 2001). Of course, for the rat and mouse, the sequence corresponds to the residues of 99-109 amino acids. In chicken and other poultry (Table 1), the corresponding region of the 98-108 residues is more conserved than in the mammals, of which, the amino acid residues, Ser^{105}-His^{106}-Asn^{107}-Gly^{108}, within the FSHβ subunit are unique to the sampled chicken and quail. The amino acid residues Ser^{105}-His^{106}-Asn^{107}-Gly^{108} in the chicken substitute Gly^{107}-Glu^{108}-Met^{109}-Lys^{110} in the human; Gly^{107}-Asp^{108}-Met^{109}-Lys^{110} in the equine; Arg^{107}-Glu^{108}-Ile^{109}-Lys^{110} in the bovine; and Ser^{107}-Asp^{108}-Ile^{109}-Arg^{110} in the ovine (Figure 3). The variation in this region probably has a strong influence on conformation of the heterodimer, or on binding with the receptor, but the mechanism by which this region has an impact on specificity in mammals is not understood (Chopineau et al., 2001). Taken together, higher conservation of this region in the chicken may be due to the critical role of the normal function of the *FSHb* gene in the hormonal specificity and regulation of reproduction. More extensive research on how the sequence functions is ongoing.

It's well known that the *FSHR* gene was selectively expressed on granulosa cells of the ovary in the female and sertoli cells of the testis in the male, but relatively little is known about the distribution of *FSHR* mRNA expression in other tissues of the chicken. In the present study, expression of *FSHR* mRNA was detected in Dagu hens at 23 weeks of age. It was found that *FSHR* mRNA was expressed in uterus, oviduct, pancreas and glandular stomach tissues (in addition to ovary), but not in the pituitary gland and kidney out of the sampled tissues in hens. This may not be completely consistent with the report (You et al., 1996) that the chicken FSHR mRNA transcript was not detected in the oviduct, liver and other tissues using Northern blot analysis. The divergence probably results from the following aspects: i) It has been reported that the expression level of non-gonadal gonadotropin (including LH/hCG) receptors is much lower than in the gonads of mammals (Ziecik et al., 2005; Ziecik et al., 2007). The expression level of chicken *FSHR* mRNA in oviduct tissue sampled at the reproductive phase was relatively lower in this study, so that it was not clearly observed by Northern blot analysis. ii) It is well accepted that the sensitivity of the method to investigate the levels of mRNA expression by Northern blot is much poor than by RT-PCR analysis. Here, the expressions of *FSHR* mRNA in the non-gonadal tissues (uterus, oviduct, pancreas and glandular stomach) in hens revealed that FSH plays a substantial role as a molecular autocrine-paracrine regulator to its non-gonadal receptors presented extensively in the chicken, which has been previously confirmed in humans and other mammals (Mizrachi, 1999; Shemesh, 2001;

Fields and Shemesh, 2004; Ziecik et al., 2005). To understand the physiological mechanism of FSH receptors in extra-gonadal tissues of the chicken, more research is required.

With the aim of studying the functions of the chicken FSHβ subunit, we constructed the pET-28a-FSHb expression vector, and it effectively expressed in *E. coli* BL21 (DE3) cells. The recombinant protein with a molecular weight of about 19 kDa was obtained with around 4 kDa of amino acid residues from pET-28a vector included, which corresponds to the target protein of the chicken FSHβ subunit at about 14.74 kDa. Clear active effects of the administered recombinant FSHβ protein on mRNA expression of the *FSHR* gene in the sampled uterus, ovary and oviduct tissues of Dagu hens were revealed, whereas no significant increases of *FSHR* mRNA expression levels in glandular stomach and kidney tissues were demonstrated, thereby confirming that the expressed FSHβ protein possessed biological activity. Although still greater understanding is needed, the present observation provides evidence for an up-regulating effect of the FSHβ protein on the expression of *FSHR* mRNA within the breed, and for a potential role in regulation of chicken reproductive physiology that may also utilize signaling mechanisms similarly to gonadal receptors as reported in mammals (Garcia-Campayo and Boime, 2001; Stewart, 2001; Fields and Shemesh, 2004).

REFERENCES

Barnes, M. H., S. D. Miller and N. C. Brown. 2002. DNA polymerases of low-GC gram-positive eubacteria: identification of the replication-specific enzyme encoded by dnaE. J. Bacteriol. 184:3834-3838.

Bousfield, G. R., V. Y. Butnev, W. J. Walton, V. T. Nguyen, J. Huneidi, V. Singh, V. S. Kolli, D. J. Harvey and N. E. Rance. 2007. All-or-none N-glycosylation in primate follicle-stimulating hormone β-subunits. Mol. Cell. Endocrinol. 260-262:40-48.

Camp, T. A., J. O. Rahal and K. E. Mayo. 1991. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Mol. Endocrinol. 5:1405-1417.

Chappel, S. C. and C. Howles. 1991. Re-evaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. Hum. Reprod. 6:1206-1212.

Choi, J. H., K. C. Choi, N. Auersperg and P. C. K. Leung. 2005. Gonadotropins upregulate the epidermal growth factor receptor through activation of mitogen-activated protein kinases and phosphatidyl-inositol-3-kinase in human ovarian surface epithelial cells. Endocr. Relat. Cancer 12:407-421.

Chopineau, M., N. Martinat, C. Galet, F. Guillound and Y. Combarnous. 2001. β-subunit 102-104 residues are crucial to confer FSH activity to equine LH/CG but are not sufficient to confer FSH activity to human CG. J. Endocrinol. 169:55-63.

Combarnous, Y. 1992. Molecular basis of the specificity of binding of glycoprotein hormones to their receptors. Endocr. Rev. 13:670-691.

Conover, W. J. 1999. Practical Non-Parametric Statistics, 3rd edition. John Wiley and Sons, New York.

Dai, H. C., L. D. Long, X. Z. Zhang, W. M. Zhang and X. X. Wu. 2007. Cloing and expression of the duck Leptin gene and effect of Leptin on intake and fatty deposition in mice. Asian-Aust. J. Anim. Sci. 20:850-855.

David, K. L. and K. P. Wright. 2007. Reproductive physiology. General Gynecology, Pages 21-41.

Esch, F. S., A. J. Mason, K. Cooksey, M. Mercado and S. Shimasaki. 1986. Cloning and DNA sequence analysis of the cDNA for the precursor of the β chain of bovine follicle stimulating hormone. Proc. Natl. Acad. Sci. USA 83:6618-6621.

Fields, M. J. and M. Shemesh. 2004. Extragonadal luteinizing hormone receptors in the reproductive tract of domestic animals. Biol. Reprod. 71:1412-1418.

Findlay, J. K. and A. E. Drummond. 1999. Regulation of the FSH receptor in the ovary. TEM 10:183-188.

Fritz, I. B. 1982. Comparison of granulose and sertoli cells at various stages of maturation: similarities and differences. Adv. Exp. Med. Biol. 147:357-384.

Garcia-Campayo, V. and I. Boime. 2001. Independent activities of FSH and LH structurally confined in a single polypeptide: selective modification of the relative potencies of the hormones. Endocrinology 142:5203-5211.

Gharib, S. D., A. Roy, M. E. Wierman and W. W. Chin. 1989. Isolation and characterization of the gene encoding the beta-subunit of rat follicle-stimulating hormone. DNA 8:339-349.

Griswold, M. D., L. Hecker and C. Linder. 1995. The molecular biology of the FSH receptor. J. Steroid Biochem. Mol. Biol. 53:215-218.

Guzman, K., C. D. Miller, C. L. Phillips and W. L. Miller. 1991. The gene encoding ovine follicle-stimulating hormone beta: isolation, characterization, and comparison to a related ovine genomic sequence. DNA Cell Biol. 10:593-601.

Han, H. M. 2006. Single nucleotide polymorphism in chicken *FSHb* gene and association with early egg production. Dissertation for master's degree. Hefei: Anhui Agricultural University. (in Chinese)

Heckert, L. L. and M. D. Griswold. 2002. The expression of the follicle-stimulating hormone receptor in spermatogenesis. Recent Prog. Horm. Res. 57:129-148.

Heckert, L. L. 2001, Activation of the rat follicle-stimulating hormone receptor promoter by steroidogenic factor1 is blocked by protein kinase A requires upstream stimulatory factor binding to A proximal E box element. Mol. Endocrinol. 15: 704-715.

Hong, K. Y. 2004. Polymorphism in *PRL*, *PRLR*, *FSHb* and *ESR* genes of chicken and association with egg production. Dissertation for master's degree. Nanjing: Nanjing Agricultural University. (in Chinese)

Jameson, J. L., C. B. Becker, C. M. Lindell and J. F. Habener. 1988. Human follicle-stimulating hormone beta-subunit gene encodes multiple messenger ribonucleic acids. Mol. Endocrinol. 2:806-815.

Kato, Y. 1988. Cloning and DNA sequence analysis of the cDNA

for the precursor of porcine follicle stimulating hormone (FSH) beta subunit. Mol. Cell. Endocrinol. 55:107-112.

Keene, J. L., M. M. Matzuk, T. Otani, B. C. J. M. Fauser, A. B. Galway, A. J. W. Hsueh and I. Boime. 1989. Expression of biologically active human follitropin in chinese hamster ovary cells. J. Biol. Chem. 264:4769-4775.

Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17:1244-1245.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Laster, D. B. 1972. Disappearance and uptake of [^{125}I] FSH in the rat, rabbit, ewe and cow. J. Reprod. Fertil. 30:407-415.

Lin, J. B., X. Chen and M. D. Liu. 2002. SPSS11.0 Statistical Analysis Actual Practice Design. China Railway Press, Beijing. (in Chinese)

Lu, W. F., J. Zhao, G. J. Wei and X. S. Shan. 2007. Cloing and prokaryotic expression of the mature fragment of the Chinese Yellow Bovine myostatin gene. Asian-Aust. J. Anim. Sci. 20:827-831.

Maurer, R. A. and A. Beck. 1986. Isolation and nucleotide sequence analysis of a cloned cDNA encoding the β-subunit of bovine follicle-stimulating hormone. DNA 5:363-369.

Maurer, R. A. 1987. Molecular cloning and nucleotide sequence analysis of complementary deoxyribonucleic acid for the β-subunit of rat follicle stimulating hormone. Mol. Endocrinol. 1:717-723.

Mizrachi, D. 1999. Follicle stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis. Biol. Reprod. 61:776-784.

Moyle, W. R., R. K. Campbell, R. V. Myers, M. P. Bernard, Y. Han and X. Wang. 1994. Co-evolution of ligand-receptor pairs. Nature 368:251-255.

Moyle, W. R. and R. K. Campbell. 1996. Gonadotropins. In "Reproductive Endocrinology, Surgery, and Techonology" (Ed. E. Y. Adashi, J. A. Rock and Z. Rosenwaks), pp. 683-724. Lippincott-Raven, Philadephia.

Rao, M. C., A. R. Midgley and J. S. Richards. 1978. Hormonal regulation of ovarian cellular proliferation. Cell 14:71-78.

Oktay, K., D. Briggs and R. G. Gosden. 1997. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human Ovarian follicles. J. Clin. Endocrinol. Metab. 82:3748-3751.

Padmanabhan, V., F. J. Karsch and J. S. Lee. 2002. Hypothalamic, pituitary and gonadal regulation of FSH. Reproduction 59:67-82.

Pierce, J. G. and T. F. Parsons. 1981. Glycoprotein hormones: Structure and function. Annu. Rev. Biochem. 50:465-495.

Ryan, R. J., H. T. Keutmann, M. C. Charlesworth, D. J. McCormick, R. P. Milius, F. O. Calvo and T. Vutyavanich. 1987. structure-function relationship of gonadotropins. Recent Prog. Horm. Res. 43:383-429.

Saneyoshi, T., K. S. Min, M. X. Jing, Y. Nambo, T. Hiyama, S. Tanaka and K. Shiota. 2001. Equine follicle-stimulating hormone: molecular cloning of beta subunit and biological role of the asparagine-linked oligosaccharide at asparagines (56) of alpha subunit. Biol. Reprod. 65:1686-1690.

Saxena, B. B. and P. Rathnam. 1976. Amino acid sequence of the beta subunit of follicle-stimulating hormone from human pituitary glands. J. Biol. Chem. 251:993-1005.

Shemesh, M. 2001. Actions of gonadotrophins on the uterus. Reproduction 121(6):835-842.

Shen, San-Tai and John Yuh-Lin Yu. 2002. Cloning and gene expression of a cDNA for the chicken follicle-stimulating hormone (FSH)-β-subunit. Gen. Comp. Endocrinol. 125:375-386.

Simoni, M., J. Gromoll and E. Nieschlag. 1997. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 18:739-773.

Stewart, E. A. 2001.Gonadotropins and the uterus: is there a gonad-independent pathway? J. Soc. Gynecol. Investig. 8:319-326.

Themmen, A. P. N. and I. T. Huhtaniemi. 2000. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr. Rev. 21:551-583.

Tisdall, D. J., K. Watanabe, N. L. Hudson, P. Smith and K. P. McNatty. 1995. Follicle stimulating hormone receptor gene expression during ovarian follicular development in sheep. J. Mol. Endocrinol. 15:273-281.

Tran, T., G. R. Bousfield and B. Shuai. 2008. Expression of a novel follicle a stimulating hormone glycoform relating to fertility. Proceedings of the 4th Annual GRASP Symposium, Wichita State University.

Lovell, T. M., P. G. Knight, N. P. Groome and R. T. Gladwell. 2001. Changes in plasma inhibin a levels during sexual maturation in the female chicken and the effects of active immunization against inhibin α-subunit on reproductive hormone profiles and ovarian function. Biol. Reprod. 64:188-196.

Ulloa-Aguirre, A. and C. Timossi. 1998. Structure-function relationship of follicle-stimulating hormone and its receptor. Hum. Reprod. Update 4:260-283.

Watkins, P. C., R. Eddy, A. K. Beck, V. Vellucci, B. Leverone, R. E. Tanzi, J. F. Gusella and T. B. Shows. 1987. DNA sequence and regional assignment of the human follicle-stimulating hormone β-subunit gene to the short arm of human chromosome 11. DNA 6:205-212.

Woods, D. C. and A. L. Johnson. 2005. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol. Reprod. 72: 643-650.

Wu, W., R. F. Xu, H. Xu and L. Xiao. 2008. Expression of the β-catenin gene in the skin of embryonic geese during feather bud development. Poult. Sci. 87:204-211.

Xu, Z. Z., H. A. Gaverick, G. W. Smith, M. F. Smith, S. A. Hamilton and R. S. Youngquist. 1995. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribo-nucleic acids in bovine follicles during the first follicular waves. Biol. Reprod. 53:951-957.

Yamamura, N., M. Takeishi, H. Goto, M. Tagami, T. Mizutani, K. Miyamoto, O. Doi and M. Kamiyoshi. 2001. Expression of messenger RNA for gonadotropin receptor in the granulose layer during the ovulatory cycle of hens. Comp. Biochem. Physiol. 129:327-337.

Yang, Y., Y. X. Huang, Q. H. Li, G. N. Ning and X. Y. He. 2007. Effects of diet energy on FSH, LH, P4 in plasma and laying rates of layers. Acta Veterinaria et Zootechnica Sinica 38:

1195-1203. (in Chinese)

Yaron, Y., D. Schwartz, M. I. Evans, J. B. Lessing and V. Rotter. 1998. Alternatively spliced mRNA transcripts encoding the extracellular domain of the FSH receptor gene: expression in the mouse ovary during the ovulatory cycle. J. Reprod. Med. 43:435-438.

You, S., J. T. Bridgham, D. N. Foster and A. L. Johnson. 1996. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol. Reprod. 55:1055-1062.

Zhang, W. B. and X. H. Chen. 2006. Cloning and expression analysis of interferon-γ-inducible-lysosomal thiol reductase gene in large yellow croaker (*Pseudosciaena crocea*). Mol. Immunol. 43:2135-2141.

Ziecik, A. J., G. Bodek, A. Biltek, M. Kaczmarek and A. Waclawik. 2005. Nongonadal LH receptors, their involvement in female reproductive function and a new applicable approach. Vet. J. 169:75-84.

Ziecik, A. J., M. M. Kaczmarek, A. Blitek, A. E. Kowalczyk, X. Li and N. A. Rahman. 2007 Novel biological and possible applicable roles of LH/hCG receptor. Mol. Cell. Endocrinol. 269:51-60.