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Original Article

Expression and localization of rat stomach follicle stimulating hormone and its receptor and analysis of core segments sequence

Yu-Tao Ma, Lian-Quan Zhou

Department of Gastroenterology, Dezhou City People's Hospital, Dezhou 253014, PR China

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Abstract: To investigate the expression and localization relationship between follicle stimulating hormone (FSH) and its receptor (FSHR) in rat stomach tissues and further determine the gene sequence characteristics in order to provide a theoretical basis for further study on gastric FSH functional regulation. Immunofluorescence double labeling was used to determine the localization of FSH and its receptor in rat stomach tissue. Situ hybridization was used to determine the distribution of expression of the protein. RT-PCR method was used to detect the content of FSH and its receptor mRNA in rat gastric tissue. The cloned gene and sequencing of the core gene cDNA fragments were obtained and analyzed. Gastric parietal cells and the main cells in rats contained the FSH receptor and immunofluorescence positive substance. Both the two have coexistence within the same cell. The positive substance was distributed in the cytoplasm and the nucleus was negative. The above mentioned cells also contained FSH and FSHR mRNA hybridization signal. The signal substance was also distributed in the cytoplasm and the nucleus was negative. FSH and its receptor gene in gastric tissue cells showed the similar expression levels by detecting the level of mRNA. By analyzing the amplified FSH and FSHR gene from rat stomach tissues with specific article by sequence, it revealed that the amplified product was fully consistent with the reported FSH gene sequences of rat; FSHR gene sequence and amplification product were exactly the same as that of rat testis tissue. FSH and its receptor distributed and expressed in the gastric parietal cells and primary cells in rats, suggesting the role of FSH on the stomach tissue may be through autocrine or paracrine to achieve.

Keywords: Follicle stimulating hormone, follicle stimulating hormone receptor, immunofluorescence, situ hybridization, RT-PCR

Introduction

FSH (follicle stimulating hormone) is the key signaling molecule of hypothalamic-pituitary-target gland axis, and is the glycoprotein hormone secreted by the pituitary. It is widely believed previously that pituitary glycoprotein hormones and their receptors exist only in specific endocrine target organs and tissues. Recent studies have found that, in addition to the hypothalamic-pituitary-gonadal axis, this hormone is also present in other tissues and organs [1], and exhibits a variety of biological activity, such as rat pancreas [2], liver [3], the hippocampus [4], bones [5, 6] and skin tissue [7]. The conclusion that these substances and their receptors exist outside endocrine tissues is proposed recently, which is still controversial. To confirm this deduction, the basis in transcription and gene molecular levels is necessary, while such experimental evidence has so far not been reported. To this end, on the basis of early experimental results [8], we have adopted double labeling immunofluorescence localization, in situ hybridization and gene sequence analysis to further study the gastric follicle-stimulating hormone and its receptor, in order to fully demonstrate the distribution and expression of FSH and its receptor outside endocrine tissues, especially in stomach tissue, providing morphological evidence for FSH regulating physiological functions of the stomach.

Material and methods

Animal and reagents

20 SD rats of either gender, body mass (200 ± 20) g, were purchased from Beijing Vital River Laboratory Animal Co., Ltd. Microscope (BA400) was purchased from Mike Audi. Trace UV spec-

trophotometer (Q-5000) was provided by US Quawell company; gel image analyzer (WD-9413) was purchased from Beijing Liuyi Instrument Factory; large horizontal electrophoresis tank (DG-3D) and bistable digital electrophoresis (DG-III) were provided by Beijing Dong Lin Changsheng biotechnology CO.LTD; ordinary PCR instrument (9600) was purchased from ABI company.

Immunofluorescence double labeling

Test specimen was processed according to the following procedure: 4% neutral formalin-fixed, paraffin-embedded, sliced; tissue dewaxing and hydration: xylene immersion for 6 min, 5 min; gradient ethanol dehydration; pure water immersion for 5 min; PBS immersion for 5 min; antigen retrieval: sodium citrate microwave antigen retrieval for 12 min. PBS washing 5 min × 3 times; serum sealing: Wipe the excess moisture and drop goat serum, sealing at room temperature for 30 min; primary antibody incubation: remove the serum on the tissues, drop FSH/FSHR antibody dilution (1:150/1:200), 4°C incubate overnight. PBS washing 5 min × 3 times; Secondary antibody incubation: wipe excess water surrounding the tissue, drop TRITC (1:50) labeled goat anti-rabbit working solution, at room temperature for 30 min. PBS wash 5 min × 3 times; wipe excess water surrounding the tissue, drop FITC (1:50) labeled goat anti-mouse working solution at room temperature for 30 min. PBS wash 5 min × 3 times; glycerine-TBS sealing. PBS instead of primary antibody was used as a negative control; positive section provided by reagent company was used as a positive control.

CFSSM observation

CFSSM (OFSympus FV300) was used for observation. The objective lens were respectively × 10 (numerical aperture 0.4) and × 20 (numerical aperture 0.7). FITC488 and Cy3548 images were obtained from dual excited 488 nm and 548 nm, with only a few signals at other wavelengths. Appropriately adjust FITC488 and Cy3 548 signals to maintain optimal experimental conditions; try to make FITC488 and Cy3 548 images accurately aligned in the same focal plane.

In-situ hybridization

The gastric tissues of above anesthetized rats were removed, fixed in 4% paraformaldehyde for 6-8 hours at room temperature, then rinsed with deionized water overnight; through gradient-ethanol dehydration, xylene transparent, paraffin-embedded, samples were sliced into 4 μm paraffin sections. The entire production process was out of RNA enzyme contamination.

Preparation of probe According to FSH and FSHR gene sequences, the DNA STAR software was used to design and screen antisense oligonucleotide probes, and then the probes were input Genebank to confirm that the probe had no homology with other genes. Probe sequence was as follows: FSH: 5'-ACAGCCAGGCAATCTTATGGTCTCGTA-3', FSHR: 5'-GTTTCTGTCCTGTAAATCTGGGCTTGC-3'; the design and synthesis of probes were completed by Beijing Dingguo changsheng biotech CO.LTD as well as digoxin-labeling.

In situ hybridization procedure Paraffin sections were conventionally dewaxed and placed into the water, and incubated with 50 mL levamisole at room temperature for 30 min; after washed with high-pressure water for 3 min × 3 times, samples were incubated with 50 μL 1 × proteinase K at 37°C for 20 min, washed with 0.05 M PBS for 3 min × 3 times, and washed with distilled water once for 3 min; pre-hybridization: drop pre-hybridization solution on each slide, incubate at 42°C for 2~4 h, remove excess liquid; the preparation of probe hybridization solution: 1 ug/μL of digoxin probe was diluted with DEPC water; denaturation was performed at 94°C for 5 min; after removed, it was placed in the ice bath immediately for 5~10 min. Then the probe/hybrid liquid (1/9) was mixed as the working fluid; 50 μL probe hybridization fluid was dropped on each slide, 42°C overnight; 37°C SSC liquid elution: 2 × SSC washing 5 min × 2, 0.5 × SSC washing 15 min × 2, 0.2 × SSC washing 15 min × 2; 0.05 MPBS washing 3 min × 3; incubation with 50 μL 1 × blocking protein solution at 37°C for 30 min were performed in order; and then shake off the excess liquid without washing. The probe was incubated with 50 ul rabbit anti-digoxin (1:100) at 37°C for 1 h, washed with 0.05M PBS for 3 min × 3 times, incubated with 50 μL AP-goat anti-rabbit IgG (1:100) at 37°C for 1 h, washed with 0.05 MPBS for 3 min/× 3 times. In NBT/BCIP color and development, the reaction was monitored by microscopy to terminate it at any time. Gradient alcohol dehydration, xylene

transparent, and neutral gum sealing were performed. With Moticcam 2306 imaging device under a microscope to get the best view picture. For negative controls, probe hybridization solution was substituted by hybridization buffer without probe.

RT-PCR

Design and synthesis of primers: Full length sequence of target gene mRNA was obtained from GenBank. Primers were synthesized by Dingguo changsheng biotech CO.LTD. Primers were as follows: Primer sequences: GAPDH, 5' ACAGCAACAGGGTGGTGGAC 3', 5' TTTGAGGGTGCAGCGAACTT 3', product length was 252 bp; FSH 5AGACCAAACACCCAGAA 3', 5' CACCGAAGGAGCAGTAG 3', product length was 193 bp, FSHR, 5' TCTGATAGATGATGAACCC 3', 5' CAAAGACAGTGAAAAAGCC 3', the product length was 413 bp.

RNA isolation: 50~l00 mg stomach tissue samples were removed from -80°C freezer and placed into a mortar, ground in liquid nitrogen for three times; a milliliter Trigol was mixed with 50~100 mg tissue, stand at room temperature for 5 min to make it fully cracking; then it was centrifuged at 12000 rpm for 5 min; the pellet was discarded; 200 μL of chloroform per milliliter Trigol was added, standing at room temperature for 15 min; after centrifugation at 4°C and 12000 rpm for 15 min, the upper aqueous phase was collected, placed into another centrifuge tube and mixed with 0.5 ml isopropanol per ml Trigol, standing at -20°C for 30 min; after 4°C 12000 rpm centrifugation for 10 min, the supernatant was discarded; RNA sink to bottom of the tube; 1 ml 75% ethanol was added, gentle shaking tube to suspend sediment. After 4°C 8000 rpm centrifugation for 5 min, the supernatant was discarded; The precipitate was dried or vacuum-dried at room temperature for 5~10 min; the RNA sample was dissolved with 30 μL TE buffer at 55~60°C for 5~10 min.

The dissolved total RNA was mixed with DEPC water to a total volume of 300 μL. 300 μL chloroform was added, mixing for 5 min; after 4°C 12000 rpm centrifugation for 10 min, the supernatant was sucked into a new DEPC-treated centrifuge tube, repeated once; after adding an equal volume of isopropanol, sample was placed at -20°C for 20~30 min and centrifuged at 4°C 12000 rpm for 10 min; the supernatant was discarded. 1 ml 75% ethanol was added after precipitation. After 4°C 8000 rpm centrifugation for 5 min, the supernatant was discarded; the precipitate was dried or vacuum-dried at room temperature for 5~10 min. DEPC-treated water was added to make RNA being purified.

Determination of RNA concentration and purity: RNA concentrations: the absorbance of 2 μL RNA sample at 260 nm was quantified with a trace UV spectrophotometer Q5000. RNA purity determination: RNA absorbance values at 260 nm and 280 nm (A260 and A280) were quantified with a trace UV spectrophotometer Q5000, recording OD260/OD280; the OD260/OD280 of all specimens was required between 1.7 and 2.0; lower than this value showed that it contained protein impurities, which should be re-purified with chloroform.

Determination of RNA integrity: After the RNA was subjected to agarose gel electrophoresis, RNA electrophoresis bands were observed in the gel imaging system; ribosomal RNA (rRNA) 28S and 18S bands were compared; if the EB color intensity was 2:1, indicating the extracted RNA was complete.

Reverse transcription

With the DNAse-treated total RNA as a template, reverse transcription was performed using TOYOBO cDNA synthesis kit.

RT-PCR analysis

In 0.5XTBE electrophoresis solution, electrophoresis was performed with 5 V/cm of voltage for 30 minutes; electrophoresis bands of PCR products were observed in the gel imaging system and photographs were archived.

Cloning and sequence analysis of PCR products

Target fragments were recovered: The target band was cut out from the agarose gel, placed in sterile centrifuge tubes, weighed, dissolved and eluted on column; after standing at room temperature for 2 min, centrifugation at 13000 rpm was performed for 1 min to collect the DNA solution. Connectivity system: T4 enzyme 3 μL,

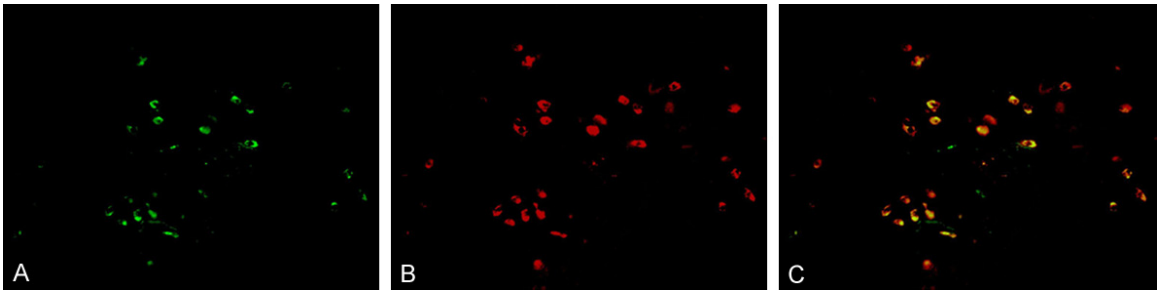

Figure 1. A. FSH-immunoreactive substances distributed in parietal cells of the stomach and the chief cells cytoplasm, nuclei are negative, positive material green fluorescence (FITC). 400 ×. B. FSHR-immunoreactive substances distributed in parietal cells of the stomach and the chief cells cytoplasm, nuclei are negative, positive material red fluorescence (Cy3). 400 ×. C. FSH and FSHR immunoreactive positive substance was distributed in the chief cells and parietal cells cytoplasm, nuclear is negative, the positive material was yellow or red and green fluorescence (FITC, Cy3). 400 ×.

the target gene product 2 μL, T carrier 1 μL, a total volume of 10 μL, 16°C overnight. And then transformation was performed: 100 μLE. coli DH5a competent cells were placed on ice to melt; the connection solution was added to the melted competent cells, placed on ice for 30 min, subjected to heat shock at 42°C for 1 min, placed on ice for 2 min; 900 μL LB solution was added, shaking-cultured at 37°C (100 rpm) for 1 h, centrifuged; bacteria was collected and 900 μL supernatant was discarded; precipitate was resuspended; the remaining 100 μL was applied to the LB plates containing 100 mmol/L ampicillin; after cultured for 12~16 h at 37°C, blue-white screening was performed; white spot was picked up and streak-cultured in LB plates containing 100 mg/L ampicillin for 10~12 h; plaque PCR identification: positive clones were selected; plasmids were extracted for electrophoresis. The positively-cloned fragments were sequenced by Beijing dingguo changsheng biotech CO.LTD.

Results

FSH and FSHR immunofluorescence test results

The expression of follicle stimulating hormone and its receptor in gastric tissue was mainly located in cytoplasm; no fluorescence reaction had been found in nucleus. The background of biopsy tissue was clear; positive FSH immunofluorescence staining was green (**Figure 1A**); positive FSHR immunofluorescence staining was red (**Figure 1B**); some cells were both FSH and FSHR positive, showing yellow or red-green interphase fluorescence (**Figure 1C**); it also had been found that FSH and FSHR immunofluorescence distributed in the same nucleus. Mucous cells were negative. Two kinds of negative control experiments showed no fluorescence. FSH color cells were fewer than normal, while FSHR color cells were more than normal; dual-labeled volume increased significantly, indicating that there were differences in the distribution or synthesis of FSH and FSHR in the above tissues.

FSH and FSHR situ hybridization

FSH and FSHR mRNA hybridization signals were blue or violet blue; the background was not colored, so contrasting was obvious; positive cells were easy to identify; the negative control test was negative. Primary cells were columnar; nuclear was round, at the bottom of the cell, non-staining; cytoplasm was blue or violet blue, especially at the top of the cell, while the base color was weakness. Parietal cells were bulky, mostly conical; nuclear was round, centered but not colored; cytoplasm showed uniform and clear blue or violet blue. FSH and FSHR mRNA hybridization signal distribution was consistent in rat gastric body; positive signals were mainly located in the cytoplasm of parietal cells and primary cells; nucleus was negative; FSH and FSHR hybridization signal close to the gastric cavity was stronger than that at the base bottom of the cells, which was similar to our previous immunohistochemical results [8]. But the hybridization signal in mucous cells was negative (**Figure 2**).

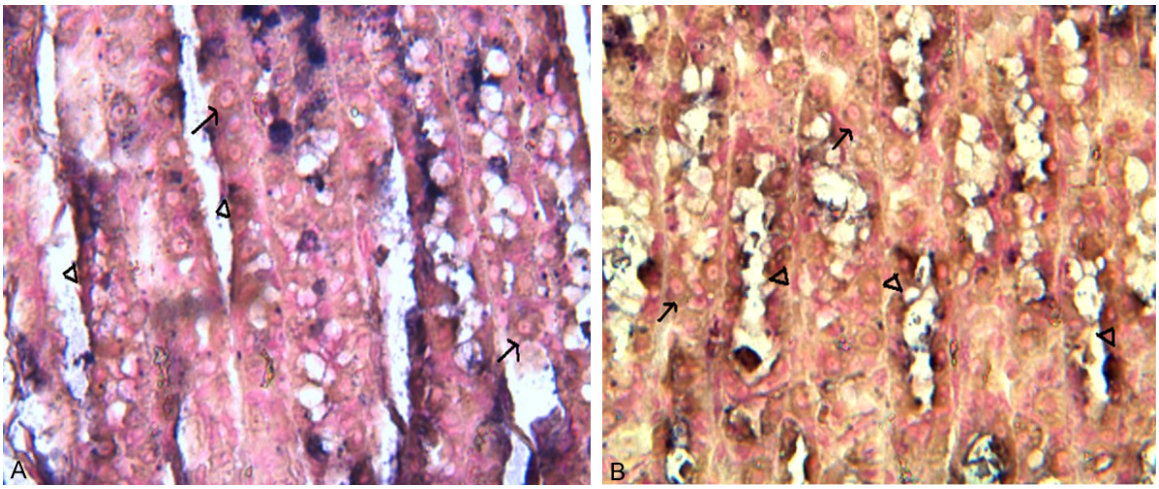

Figure 2. A. FSH hybridization signals were detected in cytoplasm of the parietal cells (↑) and chief cells (△) of fundic gland. Hybridization signals higher cell free surface. 400 ×. B. FSHR hybridization signals were detected in cytoplasm of the parietal cells (↑) and chief cells (△) of fundic gland. Hybridization signals higher cell free surface. 400 ×.

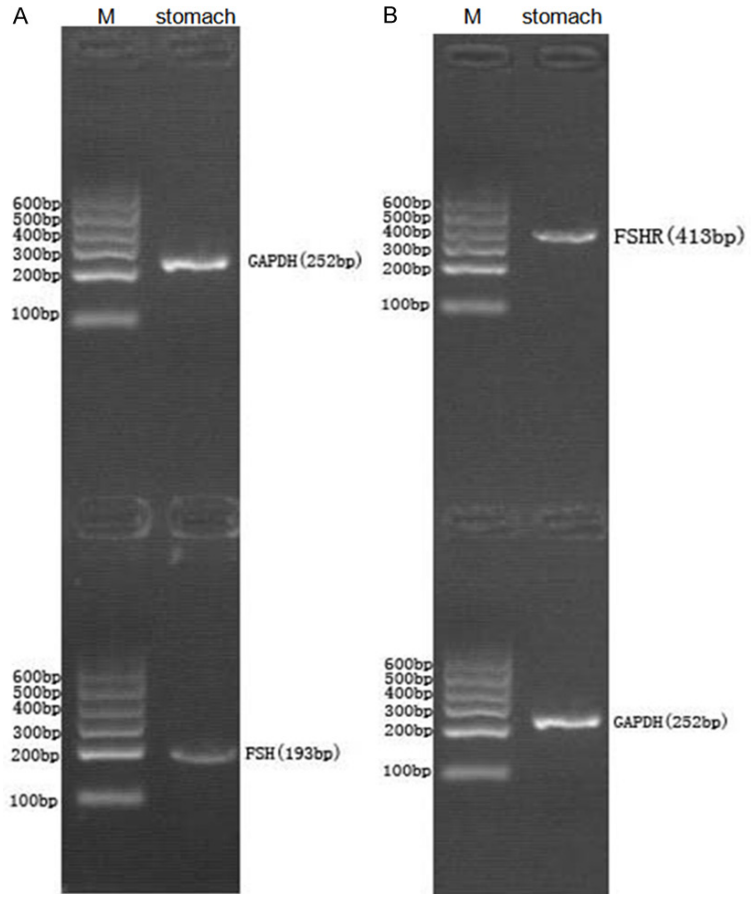

Figure 3. A. Core fragment of rat gastric FSH gene amplification product by agarose gel electrophoresis. GAPDH is a reference gene. B. Core fragment of rat gastric FSHR gene amplification product by agarose gel electrophoresis. GAPDH is a reference gene.

RT-PCR results

1% agarose gel electrophoresis and ethidium bromide staining showed that FSH and FSHR had a specific fluorescence band around 193 bp and 413 bp, respectively, consistent with the designed bases (**Figure 3**). While in the negative control group, there were no bands.

Sequencing and homology comparison of positive clones

Two positive clones of FSH and FSHR were selected and sequenced, respectively; homology search of the sequencing results was performed in GenBank; the results showed that FSH sequence was exactly the same as rat pituitary FSH gene sequence; FSHR sequence was exactly the same as the FSH gene sequence in rat testicular tissues.

Discussion

Studies on FSH and its receptor began in the 1920s; in 1920, Zondek and As-chheim

proposed that female animal pituitary possibly secreted two gonadotropic materials: one is to stimulate the follicular maturation, the other is to promote ovulation and corpus luteum formation; latter other researchers defined the two as follicle stimulating hormone (FSH) and luteinizing hormone (LH). More and more studies show that, FSH and its receptor has a broad distribution and role in the body; FSH not only regulates the reproductive functions, but also is involved in regulating other physiological activities [9]. Studies have shown that FSH can stimulate and induce the expression of proto-oncogene c-fos [10] and c-myc mRNA specifically. Due to the broad and unique biological functions, it has a very broad and attractive prospect in therapeutic areas of human-related diseases.

Biologically active substances produced by the body can regulate body functions through the endocrine, paracrine, autocrine, endocrine and neurosecretory ways. An increasing number of experiments show that autocrine is a more widespread natural phenomenon. Previous experiments found that FSH and GnRHR co-localized at gastric glands [8], preliminary indicating that the gastric parietal cells and primary cells were the target cells of FSH. This study used immunofluorescence double labeling positioning, in situ hybridization, RT-PCR and sequence analysis to comprehensively verify the distribution and expression of FSH and its receptor in gastric tissue and the structural gene sequences, and found that the gene sequences were same as those in rat pancreas [2] and submandibular gland [11], indicating FSH and its receptor distributed in the digestive organs and tissues of rats, with the same functionality. In addition to parietal cells, the distribution and expression of FSH and its receptor had also been found in the primary cells, indicating that FSH and its receptor were synthesized and secreted in the gastric parietal cells and primary cells; gastric glands could produce FSH and its receptor by autocrine way. Morphological and molecular study further confirmed that GnRH regulated gastric glands to produce FSH by autocrine or paracrine.

Parietal cell cells can secret intrinsic factor and acid and regulate acid-alkali balance; it is one of the important cells of the gastric glands; especially acid secretion is most important. Recent studies show that in addition to regulating the secretion of gastrin by directly binding to the GnRH receptor in parietal cells to inhibit acid secretion, GnRH may also promote the synthesis and release of FSH in parietal cells; the latter combined with the FSHR to further inhibit acid secretion; reduced acid secretion promoted the synthesis and secretion of gastrin in feedback, which may be an important mechanism of FSH exocrine to promote the synthesis and release of gastrin. Under the stimulation of certain food or drugs, hydrochloric acid excretion can significantly increase; the maximum output of hydrochloric acid in normal person can be up to 20-25 mmol hourly; acid secretion rate in men is higher than that in women; acid secretion rate would decrease over 50 years old. This further suggests that gastric acid secretion is associated with sex hormone levels to some extent. This experiment found that the rat gastric parietal cells in rats showed both positive in FSH immunofluorescence reaction and FSHR immunofluorescence reaction; and immunofluorescence double labeling showed the two can coexist in the same cell, and in situ hybridization found that intramural cytoplasmic hybridization signal of FSH and FSHR, suggesting that there may be a GnRH-FSH adjustment shaft to regulate the function of gastric glands; but the pathway needs to be further explored.

Primary cells has the structural characteristics of typical protein-secreting cells, with many functions; it can regulate the number of parietal cells EGF through the EGF produced by the paracrine of neighboring cells [12]; these cells secreting plasminogen were regulated by nerve endocrine system, especially the increased concentration of intracellular Ca^{2+}, cAMP and protein kinase C. Immunofluorescence double labeling discovered that FSH and FSHR coexisted in the same cell; in situ hybridization found both cytoplasmic hybridization signals of FSH and FSHR in primary cells. So FSH may regulate the function of primary cells through the paracrine action of adjacent parietal cells, or regulate the growth and function of cells by autocrine effect; its exact biological effects have yet to be confirmed by functional experiments.

In this study, double labeling immunofluorescence and in situ hybridization were comprehensively used, and the expression of FSH and

its receptor was firstly found in the cytoplasm of rat gastric parietal cells and primary cells; degenerate primer was designed; its content was measured by RT-PCR; by sequencing techniques, we found that relative content of FSH and its receptor in rat gastric tissue was substantially similar; the gene sequences of FSH and its receptor were fully consistent with those in the pituitary and testicular tissue respectively, further illustrating that rat gastric primary cells or parietal cells can synthesize and secret FSH by themselves, and they are also the target organs of FSH; the expression of FSH and its receptor genes was detected at in situ hybridization level [13] or mRNA level. It powerfully suggests that pituitary glycoprotein hormones and their receptors distribute outside the endocrine tissues, with sufficient evidence. Meanwhile it suggests that FSH may also be a gastrointestinal hormone. For the reason for its existence and mechanism of action, based on a lot of previous information we speculate that gastric glands is likely involved in or regulate the synthesis and metabolism of sex hormones, or FSH and its receptor may also play a role in regulating some functions of gastric glandular tissue. This is urgent to be confirmed in our further experiments.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lian-Quan Zhou, Department of Gastroenterology, Dezhou City People's Hospital, No. 1751, Xin Hu Street, Dezhou 253014, China. Tel: +86-0534-2637487; Fax: +86-0534-2637487; E-mail: zhoulianquanzh@163.com

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