THYROSTIMULIN, BUT NOT THYROID-STIMULATING HORMONE, ACTS AS A PARACRINE REGULATOR TO ACTIV ATE THYROID-STIMULATING HORMONE RECEPTOR IN THE MAMMALIAN OVARY

Su-Chin Sun¹, Pei-Jen Hsu¹, Fang-Ju Wu¹, Sheng-Hsiang Li², Chung-Hao Lu² and Ching-Wei Luo*¹

¹Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan. ²Department of Medical Research, Mackay Memorial Hospital, Taipei, Taiwan

Running head: Thyrostimulin-TSHR signaling in the ovary

*Corresponding author: Ching-Wei Luo, Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, 155 Li-Nong Street, Section 2, Shihpai, Taipei 112, Taiwan. Tel: 886-2-28267185; Fax: 886-2-28202449; Email: cwluo@ym.edu.tw

Thyroid-stimulating hormone receptor (TSHR), activated by either TSH or a newly discovered glycoprotein hormone thyrostimulin, plays a central role in the control of body metabolism. Interestingly, in addition to its thyroid expression, we discovered the mRNA level of THSR is periodically regulated in the rat ovary by gonadotropins. Ovarian microdissection followed by real-time PCR analysis indicated granulosa cells show the highest level of TSHR expression. Cultures of follicles and primary granulosa cells demonstrated the level of TSHR is up-regulated and dampened by the gonadotropin-driven cAMP cascade and estradiol production, respectively. Furthermore, in contrast to the negligible expression of TSH in the ovary, we also found by real-time PCR and immunohistochemical analysis that thyrostimulin is mainly expressed in oocytes. Thyrostimulin, evolving before the appearance of gonadotropins, is considered the most ancestral glycoprotein hormone. Therefore, the presence of thyrostimulin in the ovary suggests it may have a primitive function in reproduction when it activates ovarian TSHR. Next, we generated recombinant thyrostimulin protein and characterized its non-covalent heterodimeric nature. Using purified recombinant thyrostimulin, we showed the human ovarian cell line NIH:OVCAR-3 also expresses endogenous and functional TSHR. Using cultured rat granulosa cells isolated from different ovarian stages, we found that treatment with thyrostimulin increased the cAMP production and c-fos gene response significantly in the presence of gonadotropins. Thus, this study demonstrates that oocyte-derived thyrostimulin and granulosa cell-expressed TSHR comprise a novel paracrine system in the ovary, where the activity is tightly controlled by gonadotropins.

Together with follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR), thyroid-stimulating hormone receptor (TSHR) belongs to a subfamily of G protein-coupled receptors that can be activated by heterodimeric glycoprotein hormones (GPHs) (1,2). However, in contrast to FSHR and LHR, which are expressed in gonads and involved in the animal reproductive cycle (3), TSHR is mainly expressed in thyroid follicular cells and is involved in regulating the body metabolic rate (4,5). Upon TSH (also named thyrotropin) binding, activated TSHR facilitates the synthesis as well as the release of thyroid hormones through the 3'-5'-cyclic adenosine monophosphate (cAMP) cascade; this increases the expression of thyroglobulin, thyroid peroxidase and sodium/iodine symporter (5,6). Interestingly, in addition to TSH, a newly discovered glycoprotein hormone thyrostimulin, which is composed of glycoprotein α2 (GPA2) and glycoprotein β5 (GPB5) subunits, has been demonstrated to be a more potent ligand of TSHR than TSH itself (7). In contrast to TSH, thyrostimulin exhibits a different and wider distribution across many tissues, where it is suggested to act as a local but yet uncharacterized regulator (8,9). In addition to its expression in the thyroid tissues, the presence of TSHR in many other mammalian tissues has also been documented. For example, high affinity TSH binding sites and the existence of the TSHR transcript have been demonstrated in lymphocytes, brown adipose tissues and erythrocytes, implying TSHR has non-metabolic roles in immunoregulation (10), thermogenesis (11,12) and local circulatory control (13), respectively. The TSHR expression
has also been found in folliculo-stellate cells in the human anterior pituitary, where TSHR signaling seems to be regulated in a paracrine manner by thyrotroph-derived TSH (14). In addition, the TSHR expression can also be detected in a number of non-thyroidal cells such as retroocular fibroblasts (15), osteocytes (16), hepatocytes (17), neuronal cells and astrocytes (18), where the potential physio-pathological roles of TSHR still await elucidation.

TSHR is phylogenetically close to FSHR and LHR, which are crucial to the control of animal reproduction. Therefore, one may speculate that TSHR may also play a role in regulating reproductive processes. Interesting in this context, transcripts encoding TSHR have been found to be abundant in gonads of various teleosts (19-22). Recently, the ovarian expression of TSHR has also been demonstrated in mammals. Immunohistochemical staining and a cAMP assay have indicated the existence of functional TSHR in mature human granulosa cells, suggesting TSHR may participate in the regulation of ovarian function (23). The ovarian localization of TSHR protein has also been confirmed in the mature bovine corpus luteum where it has been postulated to be involved in the synthesis of thyroid hormones or the modulation of progesterone synthesis locally (24). Nevertheless, how the TSHR expression is controlled and the exact roles of TSHR in the mammalian ovary remain unclear. Therefore, in this study we characterized the regulatory pattern of TSHR during the ovarian cycle and demonstrated the existence of potential ligand pair, thyrostimulin but not TSH, in the ovary. We further generated recombinant thyrostimulin and used the protein to evaluate the paracrine action of oocyte-derived thyrostimulin on TSHR-containing granulosa cells.

**EXPERIMENTAL PROCEDURES**

**Animals**- Sprague-Dawley rats were obtained from the laboratory animal center (National Yang-Ming University, Taipei, Taiwan). For time-course analyses of the TSHR mRNA levels using a superovulation model, immature female rats (26-day old) were primed with 15 IU pregnant mare serum gonadotropin (PMSG) at 0900–1000 h and received an ip injection of 10 IU human chorionic gonadotropin (hCG) 48 h later. Rats were then killed at different time points, and the ovaries were collected for total RNA extraction. All animals were housed under a controlled humidity, temperature, and light regimen and fed *ad libitum* on standard rat chow. The rats were anesthetized and killed using CO$_2$. Animal care was consistent with institutional guidelines for the care and use of experimental animals.

**Reagents and Hormones**- McCoy’s 5a medium, L-15 Leibovitz medium, RPMI-1640 medium, L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). PMSG, hCG and human FSH were purchased from Calbiochem (La Jolla, CA). The anti-progesterone antibody, anti-FLAG M1 monoclonal antibody, anti-FLAG M1 affinity gel, progesterone, estradiol, androstenedione, 3-isobutyl-1-methylxanthine, 8-bromo-cAMP, forskolin, progesterone 3-(O-carboxymethyl)oxime:BSA-FITC conjugate and other chemicals unless noted were purchased from Sigma (St. Louis, MO). The anti-GPB5 antibody was obtained from immunized rabbits using the purified recombinant GPB5 protein from *E. coli* (7).

**Preparation of the ovarian cells and culture of granulosa cells**- For assessing the gene distribution in each ovarian compartment, the ovaries from immature rats (26-day old) were microdissected in L-15 Leibovitz medium by puncturing under a stereomicroscope (Nikon Instruments, Japan). Oocytes, granulosa cells and theca-containing shells were individually collected. The purity of granulosa and theca cells was confirmed based on the differential expression of FSHR and LHR transcripts determined by real-time PCR (25). Oocytes were further treated with 350 U/ml hyaluronidase (Sigma) to remove additional cumulus cells. Corpora lutea were isolated after hCG treatment for 72 h.

For granulosa cell cultures, the rat ovaries after the indicated treatments were punctured in L-15 Leibovitz medium. Ovarian debris and oocytes were removed, and the remaining medium containing granulosa cells was collected after low-speed centrifugation at 500 g for 10 min. Granulosa cells were dispersed by repeated washing and resuspended into McCoy’s 5a culture
medium supplemented with $10^{-7}$ M androstenedione, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mM 3-isobutyl-1-methylxanthine. Cell numbers were adjusted to 2 x 10^5 viable cells/ml.

cDNA isolation, gene amplification and quantification- To determine the c-fos transcript levels in granulosa cells, cells harvested from rats with different treatments were preincubated under serum-free conditions for 20 h before stimulated with 10 nM thyrostimulin in the presence or absence of gonadotropins for an additional 30 min. To analyze the changes in TSHR transcript level in granulosa cells, cells isolated from immature 26-day old rats were incubated with forskolin, 8-bromo-cAMP or estradiol for 12h before RNA extraction. The target tissues and treated cells were harvested and their total RNAs were extracted using the TRizol reagent according to manufacturer instructions (Invitrogen). For the first-strand cDNA synthesis, 2 μg of total RNA were reverse-transcribed using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Hanover, MD) with the oligo-dT primer.

For the semi-quantitative PCR, the primer pairs for each gene were listed as follows: rat TSHR forward, ACCAGAAGCTTGACTTACATAAG; TSHR reverse, CAGGTTCCGGATACTACTCTCATT. GPA2 forward, AGGCAGC CGTCCCAATC; GPA2 reverse, TCACTTCGCACTGTCACGTTAA. GPB5 forward, TGACGGTGAAGCTGCCTAACT; GPB5 reverse, GGACAGCCATAGGTAGGTATAG. GPA1 forward, CACGTGCTGTGTGGCCAA; GPA1 reverse, CAGTGGCAGTCCGTGTGGT. TSHβ forward, CATCTGCCTGACCATCAACA; TSHβ reverse, CCTGAGAGAGTCGTTACTTG. β-actin forward, CTGCTTGCTGATCCACATCTG; β-actin reverse, CTGCTTGCTGATCCACATCTG.

Expression and purification of recombinant human thyrostimulin proteins- The full-length human GPA2 and GPB5 cDNAs were amplified by PCR from human ovarian cDNA (CLONTECH, Palo Alto, CA). To facilitate protein purification, GPA2 was further tagged with a FLAG epitope at the N terminus by replacing the endogenous signal peptide with a prolactin signal peptide and the epitope tag. The GPA2 and GPB5 cDNAs were then subcloned into the bipromoter expression vector pBudCE4.1 (Invitrogen). Fidelity of the PCR products was confirmed by sequencing of the final construct.

To generate the recombinant thyrostimulin from mammalian expression system, transfected 293T cells were clonally selected and confirmed to express both the GPA2 and GPB5 subunit proteins using antibodies against the FLAG epitope and GPB5. Conditioned medium was then purified using an anti-FLAG M1 affinity gel (Sigma) against FLAG-GPA2. Protein purity and the biochemical characteristics were analyzed by electrophoresis using a 15% SDS polyacrylamide gel.

Immunohistochemical analyses- The ovaries from immature rats were fixed using Bouin’s fixative for 8 h before paraffin embedding. Blocks were sectioned at 8 μm thickness. Immunohistochemical analyses were performed using the rabbit polyclonal antibody against GPB5 diluted at 1:100. Substitution for the primary
antibody with the rabbit preimmune serum served as the negative control. Staining was visualized using the Vectastain ABC-AP kit following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). To neutralize specific epitope binding, the GPB5 antibody was pre-saturated with purified thyrostimulin protein (1 μM) for 2 h at room temperature. The mixtures were then used on sections instead of the GPB5 antibody alone. To eliminate any endogenous biotin background, the sections were pre-treated with Avidin-Biotin Blocking kit following the manufacturer’s instructions (Vector Laboratories) before adding the primary antibody.

Assessment of the changes of cAMP and progesterone levels. For assessing the bioactivity of purified thyrostimulin on human NIH:OVCAR-3 cells, cells were treated with increasing doses of thyrostimulin in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 mM 3-isobutyl-1-methylxanthine for 16 h. For analyzing the changes of cAMP and progesterone in granulosa cells, cells from rats after different treatments were resuspended in McCoy’s 5a medium and dispensed into 12-well plates (Corning, Corning, NY) in triplicate. Following treatment with purified thyrostimulin in the presence or absence of gonadotropins for 48 h, the conditioned media were collected and stored at -80 C until their cAMP and progesterone levels were measured. The amount of cAMP in the media was determined using the cAMP-Glo Assay kit following the manufacturer’s instructions (Promega, Madison, WI). The amount of progesterone was measured by ELISA using the specific anti-progesterone antibody (Sigma).

Data analysis- All experimental results are presented as the mean ± S.D. of triplicate cultures or samples; at least two extra experiments showed similar results. Statistical significance was determined using Student's t-test for multiple group comparisons. Significance was accepted at P< 0.05 and is indicated as * unless otherwise noted.

RESULTS

Ovarian TSHR expression and its regulation by gonadotropins. Using PCR amplification, the TSHR transcript can be detected in both the ovaries and adjacent oviducts from immature and mature rats (Fig. 1A). Interestingly, quantitative real-time PCR analysis showed that the TSHR mRNA level in the immature ovaries was 6.5-fold higher than that in the mature ovaries (Fig. 1B), which suggests the TSHR expression is regulated during ovary maturation. To demonstrate this, the ovaries from superovulated immature rats treated with PMSG followed by hCG for different intervals were collected to assess changes in the TSHR expression. Using PCR amplification, the ovarian TSHR transcripts after hCG treatment were found to be decreased (Fig. 2A). Therefore, the gonadotropin effects on the TSHR expression were further characterized using quantitative real-time PCR. As shown in Fig. 2B, the TSHR mRNA level was up-regulated during the early stages right after injection of either PMSG or hCG but then gradually decreased after injection for 6 h. The TSHR expression showed the lowest level at 48 and 96 h after hCG treatment. We subsequently analyzed the TSHR expression in the various ovarian compartments by isolating cDNA from granulosa cells, theca-containing shells, luteal cells and oocytes. As shown in Fig. 2C, the TSHR transcript was mainly present in granulosa cells and was lowest in the corpus luteum.

Mechanism underlying the modulation of TSHR levels by gonadotropins. The changes in TSHR transcript level in the superovulated rat model (Fig. 2B) suggest that ovarian TSHR expression is tightly controlled by gonadotropins. It is known that the gonadotropin actions lead to a prompt increase of cAMP levels in the ovary. These, in turn, modulate steroidogenic genes that facilitate the synthesis and release of steroid hormones, mainly estradiol, as a late response to gonadotropins (26,27). To further clarify the possible mechanisms underlying the modulation of ovarian TSHR levels by gonadotropins, the effects of cAMP and estradiol on the TSHR expression were evaluated. As shown in Fig. 3A, treatments with graded doses of forskolin or 2 mM 8-bromo-cAMP increase the TSHR transcript level in cultured immature granulosa cells. Furthermore, we isolated early antral follicles and cultured them with estradiol (10^8 M) for different time intervals. The TSHR transcript was
suppressed by 67% at 3 h after estradiol treatment and maintained this low level when incubated for longer periods (Fig. 3B). The effect of estradiol on isolated immature granulosa cells was also analyzed. Treatment with estradiol led to a dose-dependent suppression of the TSHR transcript in cultured granulosa cells (Fig. 3C).

Thyrostimulin (GPA2/GPB5) acts as a paracrine ligand for TSHR in the ovary. TSHR can be activated by two heterodimeric glycoprotein hormones, TSH and thyrostimulin. To assess whether the ovary has the endogenous ligand for ovarian TSHR, the transcript of each glycoprotein subunit, GPA1 and TSHβ for TSH, or GPA2 and GPB5 for thyrostimulin, was evaluated using the rat ovaries. The primer set for each gene has been previously tested using the rat pituitary cDNA as a positive control. Primer pairs that were capable of amplifying a single-band correct gene product (data not shown) were then used to assess the gene transcript profile in the ovary. In contrast to the negligible expression of the TSHβ subunit for TSH, PCR amplification indicated that both GPA2 and GPB5 were expressed constitutively in the ovaries treated with PMSG or with PMSG followed by hCG for different intervals (Fig. 4A). Using PCR amplification, we demonstrated both the GPA2 and GPB5 transcripts can be detected in every isolated ovarian compartment (Fig. 4B). It is known the alpha subunit genes exhibit a wider and more abundant distribution than the beta subunit genes, thus the location of functional heterodimeric glycoprotein hormone depends on the expression of restricted beta subunits (9,28). We therefore quantified the expression levels of GPB5 in different ovarian compartments. As shown in Fig. 4C, oocytes contained the highest levels, while granulosa cells showed a moderate level of the GPB5 transcript.

Interestingly, immunohistochemical staining indicated a strong GPB5 immunoreactivity in oocytes but not granulosa cells (Fig. 5A-D). Similar results were also obtained using the horseradish peroxidase system counterstained with hematoxylin (Supplemental Fig. 1). The synthesis of GPB5 protein in granulosa cells is thus likely to be low or blocked at the translational level by a yet uncharacterized mechanism. To confirm the positive GPB5 staining found in oocytes, an antigen blocking assay was further performed. As shown in Fig. 5E, the GPB5 staining was completely abolished when the antibody was preabsorbed with the purified GPA2/GPB5 heterodimer. In addition, the intensity of GPB5 staining was increased after the sample was pretreated with the avidin/biotin blocking reagents that reduce the nonspecific signal from endogenous biotin or biotin-binding molecules (Fig. 5F). It is also known that thyrostimulin is ~30-fold more potent than TSH in terms of activating TSHR (8). Therefore, these results suggest the thyrostimulin, but not TSH protein, is expressed in oocytes and acts as a paracrine factor for the TSHR signaling in the ovary.

Purification of recombinant thyrostimulin and its effects on cultured ovarian cells. In order to study the ovarian functions of thyrostimulin, conditioned media collected from transfected 293T cells stably expressing FLAG-tagged GPA2/GPB5 were used for recombinant protein purification. Following affinity purification against the FLAG tag appended to GPA2, the purity and heterodimeric property of thyrostimulin were confirmed by Coomassie blue staining and Western blotting. Under reducing conditions, the purified thyrostimulin could be separated into two subunits, namely GPA2 and GPB5, and migrated as bands at ~22 and ~18 kDa, respectively (Supplemental Fig. 2A). These two bands were confirmed to be GPA2 and GPB5 monomers using the specific antibodies against the FLAG epitope on GPA2 and the GBP5 epitope (Supplemental Fig. 2B, lane 1 and 3). To further demonstrate the non-covalent heterodimeric nature of the purified thyrostimulin, we performed a cross-linking analysis using disuccinimidyl suberate. After cross-linking, both Coomassie blue staining (Supplemental Fig. 2A, lane 4) and Western blotting (Supplemental Fig. 2B, lanes 2 and 4) revealed the presence of a heterodimer, migrating as a band at ~40 kDa.

The bioactivity of purified thyrostimulin was confirmed by the protein's ability to stimulate the cAMP production in TSHR-transfected 293T cells (data not shown). Further, to test the potency of the purified thyrostimulin and the existence of endogenous TSHR on the ovarian-derived cells, human NIH:OVCAR-3, an ovarian cell line from a patient with progressive adenocarcinoma of the
ovary (29), was selected. Treatments with purified thyrostimulin stimulated the cAMP production in a dose-dependent manner. PCR amplification also showed the presence of TSHR transcript in NIH:OVCAR-3 cells (Supplemental Fig. 2C). These results confirm that the recombinant thyrostimulin is bioactive and the existence of functional TSHR in some ovarian cells.

Based on the TSHR expression pattern in the ovary, TSHR is abundant in rat granulosa cells and shows higher transcript levels after injection of gonadotropins for 6 h (Fig. 2). In consideration of the gonadotropin effects and the potential delay in TSHR translation, granulosa cells from untreated rats, rats primed with PMSG for 12 h and rats primed with PMSG for 48 h followed by hCG injection for 12 h were harvested and treated with either buffer or thyrostimulin in the presence or absence of gonadotropins (Fig. 6). In contrast to the negligible effect on cells treated with thyrostimulin alone, we demonstrated that thyrostimulin was able to augment the cAMP production (Fig. 6A-C) and c-fos gene transcription (Fig. 6D-F) to a statistically significant level when the cells were co-treated with gonadotropins, suggesting the expression of functional TSHR protein is gonadotropin-inducible. However, the progesterone production was not affected by the thyrostimulin treatments except for a slight increase when PMSG-primed granulosa cells were co-treated with FSH (Fig. 6G-I). This phenomenon, which will be discussed later, is likely to be a result of the tight control of the ovarian TSHR expression by gonadotropins.

DISCUSSION

To our knowledge, this is the first report that demonstrates the co-existence of TSHR and its cognate ligand thyrostimulin in the mammalian ovary, where they comprise a novel paracrine system. Taken together with our results, a model regarding the regulatory control of TSHR and the action of thyrostimulin in the ovary is presented and shown in Fig. 7. We have shown that thyrostimulin but not TSH is located in oocytes where it can be secreted and act as a local paracrine factor to activate the cAMP cascade and nuclear c-fos response on granulosa cells through TSHR. Contrary to the constitutive expression of thyrostimulin in the ovary, the TSHR transcript is tightly regulated during ovarian development. At the early stages of gonadotropin actions, either the transcript or the functional protein of TSHR is up-regulated. Such an up-regulation is likely to be triggered by an increase in the secondary messenger cAMP derived from activated gonadotropin receptors. This hypothesis is further supported by our results showing that there is an increase in the TSHR transcriptional levels of granulosa cells when treated with forskolin or 8-bromo-cAMP (Fig. 3A). Interestingly, a functional cAMP-response element between -139 and -131bp upstream of the translational initiation site of human TSHR gene has also been characterized and is known to be conserved in mammals (30). It is known that an increase in gonadotropins will stimulate follicle growth and luteinization, which is accompanied by a significant increase in the estradiol level. Using cultured rat granulosa cells treated with estradiol, we also showed that estradiol is likely to be the major regulator that dampens ovarian TSHR expression (Fig. 3C). Such a result is consistent with the markedly decreased in the TSHR transcript observed at the late stages of gonadotropin treatments in the superovulation model (Fig. 2B). Taken together, we conclude that the ovarian TSHR level and signaling is tightly controlled by gonadotropins through the cAMP and steroid feedback loop. This implies that the induction of functional TSHR expression is periodic and transient during ovarian development. Furthermore, the down-regulation of TSHR expression by gonadotropin-driven estradiol production also explains why there is only less than 50% of cAMP and c-fos augmentation and no apparent difference in the progesterone production during long-term treatments with thyrostimulin in gonadotropin-primed granulosa cells (Fig. 6). Alternatively, the TSHR signaling may serve to modulate different ovarian functions other than the steroidogenesis. To characterize these, a more complete inventory of TSHR function with the elimination of estradiol interference will be needed in order to understand the exact roles of TSHR in folliculogenesis and luteinization.

In addition to our results showing the regulation of TSHR transcript during the mammalian ovarian cycle, the TSHR expression in fish ovaries has also been found and
demonstrated to change significantly during the reproductive season. The ovarian expression of TSHR in channel catfish remains low throughout vitellogenesis, but is markedly elevated at the spawning period and then gradually decreases afterwards until the next run of ovarian recrudescence (22). A similar expression pattern of ovarian TSHR has also been reported in European sea bass (21), suggesting that TSHR could participate in active vitellogenesis and in the regulation of gamete maturation and ovulation.

In addition, the TSHR expression has also been found in the fish testis. In European sea bass, the TSHR transcript in the testis increases and remains high during recrudescence but declines and reaches its lowest level at the post-spawning stage, suggesting that TSHR in the testis is involved in the processes of gonadal development and/or spermiation (21). It is known that TSHR has the same evolutionary origin as FSHR and LHR and that the ligands of these proteins are all glycoprotein hormones. Therefore, the findings of the TSHR expression in both the testis and ovary suggest TSHR, like FSHR and LHR, may play a direct role in the regulation of gonadal physiology.

Recent genomic analysis has indicated that glycoprotein hormones co-evolved with their receptors and that their presence can be traced back to invertebrates (2,28). Glycoprotein hormones are composed of a common α-subunit GPA1 and a dissimilar β-subunit such as FSHβ, LHβ, chorionic gonadotropin β and TSHβ. In addition, two newly discovered glycoprotein subunits GPA2 and GPB5 were found to form thyrostimulin (7). Amphioxus, a member of the phylum Chordata, is considered to be in an evolutionary lineage between invertebrates and vertebrates and this organism has been reported to have no other glycoprotein hormone except for thyrostimulin (31). This evolutionary analysis indicates that the two heterodimeric glycoprotein subunits, GPA2 and GPB5, which form thyrostimulin, are likely to be the most ancient origins of the gonadal and thyroid glycoprotein hormones. They may have appeared in the early Cambrian period. Furthermore, in Drosophila melanogaster, a pair of glycoprotein genes, fly GPA2 and fly GPB5, has been reported and shown high homology with mammalian GPA2 and GPB5 (32). Fly GPA2 is capable of forming a heterodimer with fly GPB5 to activate a fly receptor, the Drosophila leucine-rich repeat-containing G protein-coupled receptor-1, which shows high homology to mammalian TSHR. No other pair of gonadotropin-like molecules and receptors is found in fly. These results together with the study in amphioxus strongly support the idea that the GPA2 and GPB5 genes, evolving before the appearance of the gonadotropin genes, are indeed the ancient origins of glycoprotein hormones. Therefore, one may also speculate that the primitive roles of thyrostimulin-like molecule may also involve the reproductive control and gonadal development in addition to the regulation of the metabolic rate.

From the viewpoint of an in vitro bioactivity assay, thyrostimulin is likely to serve as an alternate ligand of TSH for activating TSHR. However, its exact roles are still unknown. Patients with lost-of-function mutations of TSHβ exhibit congenital central hypothyroidism (33,34), whereas GPB5-null mice show no overt thyroid-related phenotype (8,35). Distinct from TSH expressed in the thyrotrophs, the thyrostimulin expression in the anterior pituitary is located at the corticotrophs, where its release has been demonstrated not to be in response to thyrotropin-releasing hormone (8,9). These results indicate that thyrostimulin is unlikely to be involved in the thyrotropin-releasing hormone-TSH-thyroid hormone feedback loop and substitute for the TSH activity in animals. It is known that the corticotrophs produce adrenocorticotropic hormone, which is secreted in response to the release of hypothalamus-derived corticotropin-releasing hormone and acts on the adrenal gland under stress conditions. The expression of thyrostimulin in the corticotrophs suggests thyrostimulin may also be controlled through the hypothalamus-pituitary-adrenal axis. Interestingly, the expression of TSHR has also been found in the adrenal gland (36). Future experiments will be critical to clarify whether thyrostimulin responds to the corticotropin-releasing hormone stimulation and thus plays a non-thyroidal role in the control of homeostasis or immune suppressive effects.

Though activating the same receptor as TSH, the receptor binding characteristics of thyrostimulin are quite different from those of TSH. Binding and competition assays indicate that
there are two ligand binding sites on TSHR. One site can interact with either thyrostimulin or TSH, whereas the other site can only bind thyrostimulin alone (8). The Scatchard plot for the iodinated-thyrostimulin binding fits a two-site model. The calculated $k_d$ is $1.19 \pm 0.68$ nM for the high-affinity site and $2.7 \pm 2.2$ mM for the low-affinity site. In contrast, the $k_d$ for the iodinated-TSH binding is estimated at 41 nM. This indicates that thyrostimulin is a more potent ligand than TSH. Such a result is also supported by Nakabayashi et al. (7), who showed that thyrostimulin can stimulate the cAMP production more efficiently than TSH in cells expressing recombinant TSHR.

Unlike TSH, which is mainly expressed in the anterior pituitary of mammals, the existence of thyrostimulin has been found in many different tissues such as anterior pituitary, skin, retina, adrenal gland, pancreas, central nervous system and testis (8,37), suggesting that thyrostimulin may act as a local regulator rather than an endocrine hormone. Our results also indicate that thyrostimulin but not TSH can be expressed in the rat oocytes as a paracrine factor for TSHR-expressing granulosa cells. Though we can not rule out an endocrine effect of TSH on ovarian TSHR, taken together with the ligand binding data described above, which show the bioactivity of thyrostimulin is ~30-fold more potent than that of TSH (8), one may discard the possibility of TSH interference with the paracrine action between thyrostimulin and TSHR in the ovarian compartments. Surprisingly, in situ hybridization results have indicated that the amphioxus GPB5 transcript is abundant in pre-vitellogenic oocytes (31), which also supports the hypothesis that thyrostimulin may have a primitive function in the invertebrate ovary. In addition to the ovary, several studies also suggest thyrostimulin and TSHR are possibly co-expressed in the vertebrate testis (8,19,21,37). Future experiments are needed to clarify their compartmental distribution and their signal transduction effect. These will provide a more comprehensive understanding of these proteins’ paracrine and/or autocrine activity in testicular development and spermatogenesis.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: Thyroid-stimulating hormone receptor, TSHR; follicle-stimulating hormone receptor, FSHR; luteinizing hormone receptor, LHR; glycoprotein hormones, GPHs; glycoprotein α2, GPA2; glycoprotein β5, GPB5; pregnant mare serum gonadotropin, PMSG; human chorionic gonadotropin, hCG; 3'-5'-cyclic adenosine monophosphate, cAMP.
FIGURE LEGENDS

Fig. 1 TSHR is expressed in the ovary and oviduct.
cDNAs from immature (26-day old) and mature (8-week old) rat tissues were used for (A) PCR
amplification and (B) real-time quantification of the TSHR expression levels. Data are expressed as the
mean ± S.D. Levels of β-actin serve as loading and normalized controls.

Fig. 2 Expression and regulation of TSHR in the ovary.
Rats at 26 days of age were treated with PMSG, followed 48 h later with hCG. The ovaries collected at
the indicated intervals were used for cDNA preparation. The TSHR transcripts were compared by (A)
PCR amplification and (B) real-time PCR quantification. (C) Comparison of the TSHR expression in
different ovarian cell types by real-time PCR quantification. Granulosa cells (GC), theca shells (TS) and
oocytes (OC) were isolated from the rat (26-day old) ovaries, whereas corpora lutea (CL) were obtained
from PMSG-primed rats at 72 h after hCG treatment. Data are expressed as the mean ± S.D. For all data,
at least three individual repeats were done and the β-actin levels serve as internal and normalized
controls.

Fig. 3 Forskolin, 8-bromo-cAMP and estradiol effects on the TSHR transcript in the ovary
To evaluate the cAMP effects on the TSHR expression, (A) granulosa cells from 26-day old rats were
treated with graded doses of forskolin or 2mM 8-bromo-cAMP (Br-cAMP) for 12 h. To test the estradiol
effect, (B) early antral follicles isolated from immature rats were incubated in the presence of 10-8 M
estradiol for different intervals and (C) granulosa cells from 26-day old rats were cultured in the presence
of graded doses of estradiol for 12 h. After reverse transcription, the TSHR transcript levels were
determined by real-time PCR and normalized using β-actin levels. Data were obtained from triplicate
experiments and are shown as the mean ± S.D. (*, P < 0.05).

Fig. 4 Thyrostimulin but not TSH is expressed in the ovary.
(A) The ovaries collected at indicated time intervals from superovulated rats were used for cDNA
preparation. The subunit genes, GPA1 and TSHβ for TSH, or GPA2 and GPB5 for thyrostimulin, were
PCR amplified. To assess the transcript levels of GPA2 and GPB5 in various ovarian compartments,
cDNAs from different ovarian cell types were isolated for (B) PCR amplification of the GPA2 and GPB5
genes and (C) real-time quantification of the GPB5 gene. Data are expressed as the mean ± S.D. For all
data, at least three individual repeats were done and β-actin levels serve as internal and normalized
controls. GC: granulosa cells; TS: theca shells; OC: oocytes; CL: corpora lutea.

Fig. 5 Distribution of the thyrostimulin protein in the ovary.
Ovarian sections from immature rats (26-day old) were incubated under different conditions as indicated
below. (A and B): normal rabbit serum at different magnifications; (C and D): anti-GPB5 antibody at
different magnifications; (E): anti-GPB5 antibody pre-neutralized with the purified thyrostimulin protein;
(F): an additional avidin-biotin blocking step to quench the endogenous nonspecificity of biotin
background was added before incubation with the anti-GPB5 antibody. GC: granulosa cells; TC: theca
cells; OC: oocytes. Bar: 100 μm.

Fig. 6 Effects of thyrostimulin on cAMP, c-fos gene and progesterone biosyntheses in granulosa cells
treated with or without gonadotropins
Granulosa cells from immature rats (left panel), rats injected with PMSG for 12h (central panel) or PMSG
for 48h followed by hCG for 12h (right panel) were treated with thyrostimulin (indicated as A2/B5) in
the presence or absence of gonadotropins as indicated. For the cAMP (A, B and C) and progesterone assays
(G, H and I), cells were incubated for additional 48 h before measurements. For c-fos gene induction (D,
E and F), cells were serum-starved for 20 h before thyrostimulin and gonadotropin treatments for additional 30 min. Cells were then collected for cDNA preparation. The c-fos gene levels were quantified by real-time PCR and normalized against the β-actin expression level. At least three individual repeats were done. Data were obtained from triplicate experiments and are shown as the mean ± S.D. (*, P < 0.05).

**Fig. 7 Paracrine action and regulatory mechanism of the thyrostimulin-TSHR signaling in the ovary**

The oocyte-derived thyrostimulin and granulosa cell-expressed TSHR comprise a paracrine system in the ovary, where its action is tightly controlled by gonadotropin surges.
Figure 1

(A) 

<table>
<thead>
<tr>
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<th>Ovary</th>
<th>Oviduct</th>
<th>Ovary</th>
<th>Oviduct</th>
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<tbody>
<tr>
<td>TSHR</td>
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<tr>
<td>β-Actin</td>
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</tbody>
</table>

Immature | Mature

(B) 

Relative expression

TSHR/β-Actin, x10^4

Immature | Mature
Figure 2

(A)

![Image of gel electrophoresis showing TSHR and β-actin bands for PMSG_0h, PMSG_48h, hCG_48h, and hCG_96h samples in Ovary.]

(B)

![Bar graph showing relative expression of TSHR/β-actin (×10^4) over time (h) for PMSG and hCG samples.]

(C)

![Bar graph showing relative expression of TSHR/β-actin (×10^4) for GC, TS, OC, and CL samples.]
Figure 3

(A) TSHR/β-actin, fold

- C
- 10, 30, 100 µM Forskolin
- 2 mM Br-cAMP

(B) TSHR/β-actin, fold

Estradiol treatment (h)

0, 3, 12, 24, 48 h

(C) TSHR/β-actin, fold

Estradiol treatment (M)

0, 1x10^-8, 3x10^-8 M
Figure 6

(A) Immature P=0.06

(B) PMSG 12h *

(C) hCG 12h *

(D) Immature P=0.08

(E) PMSG 12h *

(F) hCG 12h *

(G) Immature

(H) PMSG 12h *

(I) hCG 12h
Thyrostimulin, but not thyroid-stimulating hormone, acts as a paracrine regulator to activate thyroid-stimulating hormone receptor in the mammalian ovary
Su-Chin Sun, Pei-Jen Hsu, Fang-Ju Wu, Sheng-Hsiang Li, Chung-Hao Lu and Ching-Wei Luo

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