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Novel Expression of Gonadotropin Subunit Genes in Oocytes of the Gilthead Seabream (*Sparus aurata*)

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It is widely believed that FSH and LH, which are known to play key roles in controlling the production of functional oocytes in vertebrates, are synthesized and secreted exclusively by the anterior pituitary. Here we present evidence for the novel expression of FSHβ, LHβ, and the common glycoprotein-α (*Cgα*) in the gilthead seabream ovary. Using *in situ* hybridization and immunocytochemistry, FSHβ was detected in primary-growth and secondary-growth-I oocytes, LHβ was found in secondary-growth oocytes, and *Cgα* was observed in both primary and secondary-growth oocytes. Northern blot analyses demonstrated that *Fshβ* transcript is 0.6 kb in both pituitary and ovary, whereas the ovarian *Lhβ* transcript (1.1 kb), unexpectedly, is longer than the known pituitary *Lhβ* transcript (0.6 kb). Sequence analyses revealed that ovarian *Lhβ* is driven by a different promoter than pituitary *Lhβ*, which generates an additional 459 bases at the distal portion of the 5′-untranslated region of the ovarian *Lhβ*. Furthermore, using *in vitro* ovarian fragment incubation, we demonstrated that mammalian GnRH analog agonist enhanced the expression of ovarian *Fshβ* (up to 2.7-fold), *Lhβ* (up to 1.4-fold), *Cgα* (up to 1.8-fold), and the secretion of ovarian LH (up to 2.2-fold). In contrast, GnRH antagonist, analog E, suppressed the secretion of ovarian LH. Our findings suggest that a GnRH-gonadotropin axis is present in the gilthead seabream ovary and that FSH and LH, the well-characterized pituitary hormones, may have prominent novel roles in teleost intraovarian communication between oocytes and ovarian follicle cells. (*Endocrinology* 145: 5210–5220, 2004)

THE HYPOTHALAMIC-PITUITARY-GONADAL (HPG) axis is a fundamental component of the endocrine control of gametogenesis in which FSH and LH are integral endocrine messengers. In response to hypothalamic GnRH, pituitary FSH and LH are secreted into the circulation. Via the blood, they reach the gonad and bind to their respective G protein-coupled receptors, FSH receptor and LH receptor, on the ovarian follicle cells to coordinate the complex process of oocyte development. From studies of genetically derived reproductive defects in humans and mouse models, we have learned about the essential roles of FSH and LH in female reproduction (1–5). FSH acts on the granulosa cells to stimulate the activity of the aromatase system, which catalyzes the conversion of androgens into estrogens as well as to enhance the expression of LH receptor; LH plays a key role in thecal cell differentiation and promotes androgen production by these cells (6). In the late stages of follicular development, the action of LH on granulosa cells leads to the termination of their differentiation, induces oocyte meiotic maturation, and triggers follicular rupture (7, 8).

Oocyte development in teleosts can be divided into two major processes, follicular growth and final oocyte maturation (FOM). Follicular growth consists of two phases, the primary-growth (PG) phase and the secondary-growth (SG) phase. In the SG phase, oocytes undergo rapid growth associated with the uptake and accumulation of lipid and vitellogenin, *i.e.* vitellogenesis. Upon completion of the SG phase, the fully grown follicle is ready to undergo FOM, a series of developmental events preparing the oocyte for ovulation and fertilization. Pituitary gonadotropins are hormones of primary importance that trigger these two major processes. The actions of pituitary gonadotropins, however, are not direct but rather are mediated by the follicular steroids, estrogens for oocyte growth, and maturation-inducing hormone (MIH) for oocyte maturation (9).

It has long been recognized that oocyte development is supported by the ovarian follicle cells, which not only mediate the FSH and LH signals from the endocrine system but also regulate the progression of oocyte development through autocrine and paracrine pathways and gap junctions. The communication between oocytes and their companion follicle cells is essential for successful development of a functional egg (10). Literature published since Pincus' initial report in 1935 (11) lays a scientific foundation based primarily on unidirectional follicle cell-to-oocyte communication. However, findings obtained over the last decade have given rise to the more modern perspective that the intraovarian communication between oocyte and ovarian follicle cells is bidirectional (10, 12). In one significant study, the transfer of midsized oocytes (isolated from secondary follicles) back to primordial follicles doubled the rate of follicular development and the differentiation of follicle cells (13). Thus, oocytes may dominate the intraovarian communication during folliculogenesis.

In light of our growing body of information on bidirectional communication within the ovarian follicles, the immediate challenges are the search for new factors involved in this process and a detailed understanding of the mechanisms of their actions. In this study, we present evidence for the

Abbreviations: *Cgα*, Common glycoprotein-α; Dig, digoxigenin; FOM, final oocyte maturation; HPG, hypothalamic-pituitary-gonadal; ISH, *in situ* hybridization; mGnRH-A, mammalian GnRH analog; ORF, open reading frame; PG, primary growth; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SG, secondary growth; SSC, saline sodium citrate; UTP, uridine 5-triphosphate.

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novel expression of gonadotropin subunit genes in oocytes of the gilthead seabream, thus suggesting that these well-characterized endocrine regulators of folliculogenesis may also have autocrine and paracrine roles in the intraovarian communication.

Materials and Methods

Animals and sample collection

Experimental fish were held in our Aquaculture Research Center and exposed to simulated natural photoperiod conditions, 15-h light in summer and 7-h light in winter. Water temperatures ranged from 15 C (winter) to 23 C (summer), which mimics the conditions fish experience in the wild. Fish were anesthetized in 200 ppm 2-phenoxyethanol. Tissues were removed and immediately frozen in liquid nitrogen or fixed in phosphate buffered 4% paraformaldehyde solution. All animal husbandry and experimentation were conducted in accordance with our Institutional Animal Care and Use Protocols and adhered to the National Research Council's Guide for Care and Use of Laboratory Animals.

Oligonucleotide primers and sequence analyses

The oligonucleotide primers used in this study are listed in Table 1. Nucleotide and peptide sequences were aligned by the CLUSTAL W method (14). These analyses were performed with the Internet server of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). Putative transcriptional factor binding sites were located on the 5′-flanking region of *Lhβ* using analyses posted on two Web sites: http://pdap1.trc.rwcp.or.jp/ and http://www.motif.genome.ad.jp/.

RT-PCR analyses

Total RNA and mRNA isolated from samples were reverse transcribed into cDNA using random hexamers and Muloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Aliquots of these reactions served as templates in PCR amplifications of 25 μl containing 200 μM deoxynucleotide triphosphate and 0.2 μM of each specific primer [PFBF and PFBR for *Fshβ*; WTAQ13 and PLBR for *Lhβ*; PCAF and PCAR for the common glycoprotein-α (*Cgα*); LHBF1 and LHBR1 for ovarian *Lhβ*]. PCR conditions were 40 cycles of 94 C for 20 sec, 57 C for 20 sec, and 72 C for 1 min. Ten microliters of each PCR was electrophoresed through a 1.8% agarose gel. Negative controls were included in which reverse transcriptase was omitted from the cDNA synthesis reactions.

Syntheses of RNA standards and riboprobes

For RNA standard syntheses, plasmids containing *Fshβ*, *Lhβ* (15), or *Cgα* (GenBank accession no. AF300425) cDNA were linearized and used as templates for gene-specific RNA standard syntheses using an SP6/T7 transcription kit (Roche, Indianapolis, IN). RNA standards were purified through a size exclusion column (Chroma Spin-200; BD Biosciences, Palo Alto, CA), and the amount of each RNA standard was determined using a RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR). The same protocol described above was followed for riboprobe syntheses except a digoxigenin (Dig)-uridine 5-triphosphate (UTP) mixture (Roche) for *in situ* hybridization (ISH) or a ^{32}P-UTP mixture for Northern blot analyses was used instead of UTP.

Quantification of gene expression at transcript levels

Gene expression of *Fshβ*, *Lhβ*, and *Cgα* was determined at the transcript level using real-time fluorescence-based quantitative PCR assays (called quantitative PCR hereafter). *In vitro*-synthesized *Fshβ*, *Lhβ*, and *Cgα* RNA standards and total RNA, isolated from each sample, were reverse transcribed into cDNAs as described above. PCR was carried out via ABI Prism 7700 sequence detection system using SYBR Green PCR core reagent (Applied Biosystems, Foster City, CA) and gene-specific primers, WTAQ11 and -12 for *Fshβ*, WTAQ13 and -14 for *Lhβ*, and WTAQ15 and -16 for *Cgα*. Copy number in unknown samples was determined by comparing threshold cycle values (16) with the corresponding RNA standards. Values were normalized to the amount of 18s RNA (amplified by TSB18SF and TSB18SR primers) in each sample.

Northern blot analysis

Total RNA (2 μg) from seabream pituitary and mRNA (10 μg) from vitellogenic ovary were electrophoresed through a 1.2% agarose gel containing formaldehyde and transferred onto a nylon membrane. For prehybridization, the membrane was incubated with hybridization buffer I [50% formamide, 5× Denhart solution, 5× saline sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS), and 100 μg/ml yeast RNA] for 3 h at 60 C and then hybridized for 16 h with a ^{32}P-labeled antisense riboprobe at a final concentration of 1×10^6 dpm/ml. The riboprobe corresponded to either bases 18–468 of the pituitary *Fshβ* cDNA or bases 1–465 of the pituitary *Lhβ* cDNA. After hybridization, the membrane was washed for 30 min in 2× SSC, 0.1% SDS at 68 C, 30 min in 0.5× SSC, 0.1% SDS at 68 C, and a final wash of 30 min in 0.1× SSC, 0.1% SDS at 68 C. The membrane was exposed to a phosphor storage screen and visualized with a Storm 840 phosphor image analyzer (Amersham Biosciences, Piscataway, NJ).

TABLE 1. The oligonucleotide primers used in this study

Primer	Oligonucleotide sequence (5′–3′)	Gene/transcript
WTAQ11	TCATGGCAGCAGCGCTACT	*Fshβ*
WTAQ12	CACTGTCCTGCACATATGGTTGT	
PFBF	GTTCAGAGAGTGACAGAGGAAAC	
PFBR	AGATGATAAGTAACAGTATAATATAGATAGGTT	
WTAQ13	TCTTTCTGGGAGCCTCACCTT	*Lhβ*
WTAQ14	CTTGGGACAGCCCTCCTTCT	
LHBF1	TGATGACTTTATGACCCTGCAAA	
LHBR1	CAGCCGTCATCCTCTCTGTGAT	
LHBR2	CTGTGATGTGCAGGCGGAGT	
LHBR3	TTCAACTGTGAAAGCAGGAAGTGTA	
LHBR4	GCAGGAAGTGTACAGGTGCCATT	
PLBF	CGCCTGCACATCACAGAGA	
PLBR	ACAACAATGGGTGCACAGTTTCT	
WTAQ15	GACGATGACGATCCCGAAGA	*Cgα*
WTAQ16	TGTGGTTCCTCACCCTTATGC	
PCAF	TAACATGGTAACTGCTGGAATCA	
PCAR	AATTGTCTCCGGTTCCCATCT	
TSB18SF	ACCACCCACAGAATCGAGAAA	18s RNA
TSB18SR	GCCTGCGGCTTAATTTGACT	

Isolation of ovarian Fshβ and Lhβ cDNAs and the 5′-flanking region of Lhβ gene

For rapid amplification of cDNA ends (RACE) cDNA preparation, 2 μg of ovarian mRNA was used for constructing 5′- and 3′-RACE cDNA using the SMART RACE cDNA amplification kit (BD Biosciences) according to the manufacturer's instructions. Gene-specific primers, WTAQ11 for *Fshβ* 3′-RACE, WTAQ12 for *Fshβ* 5′-RACE, WTAQ13 for *Lhβ* 3′-RACE, and WTAQ14 for *Lhβ* 5′-RACE and the universal primer mix primer (adaptor primer from kit) were used for RACE amplifications. PCR products were separated on 1.2% agarose gel, purified by QIAquick gel extraction kit (Qiagen, Valencia, CA), cloned into pGEM-T (Promega) vector, and sequenced.

To use a PCR-based method for the isolation of the 5′-termini of the *Lhβ* genes, genomic DNA extracted from seabream blood cells was constructed into a GenomeWalker library (Universal GenomeWalker kit; BD Biosciences) according to the manufacturer's instructions. The first genome walking PCR was carried out using LHBR1 primer and Ap1 primer (adapter-specific primer 1 from kit) followed by a nested second amplification with LHBR2 and Ap2 primers. The second genome walking PCR was carried out using LHBR3 and Ap1 primers followed by a nested second amplification with LHBR4 and Ap2 primers. The PCR amplicons were subcloned and sequenced.

ISH

A published procedure (17) was modified and used to localize *Fshβ*, *Lhβ* and *Cgα* transcripts within the ovarian tissues. The 6-μm sections were deparaffinized in xylenes, rehydrated in a graded ethanol series, treated with proteinase K [10 μg/ml in 50 mM Tris-HCl (pH 7.5) and 50 mM EDTA] for 10 min at 37 C, and acetylated for 10 min in 0.1 M triethanolamine-HCl /0.25% (vol/vol) acetic anhydride. For prehybridization, each section was covered with 500 μl of hybridization buffer II (50% formamide, 50 μg/ml yeast tRNA, and 50 μg/ml denatured calf thymus DNA in 5× SSC) and incubated for 2 h at 58 C. Prehybridization buffer was replaced with new hybridization buffer II containing 400 ng/ml of a denatured Dig-labeled riboprobe corresponding to bases 22–393 of the pituitary *Fshβ* cDNA, bases 13–376 of the pituitary *Lhβ* cDNA (15), or bases 22–330 of pituitary *Cgα* cDNA (AF300425) and incubated overnight at 58 C. After hybridization, the sections were washed for 30 min in 2× SSC at 65 C, 30 min in 0.5× SSC at 65 C, 30 min in 0.1× SSC at 65 C, and equilibrated for 10 min in buffer I [100 mM Tris-HCl and 150 mM NaCl (pH 7.5)]. After a 30-min incubation in blocking buffer A [5% lamb serum and 2% blocking reagent (Roche) in buffer I], sections were then incubated for 2 h with 150 mU/ml alkaline phosphatase-coupled anti-Dig antibody (Roche) in buffer I containing 0.5% blocking reagent. Excess antibody was removed by two 15-min washes with buffer I and equilibrated for 5 min in buffer II [100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$ (pH 9.5)]. Color development was performed using BM AP-Purple Substrate (Roche), and sections were examined under a light microscope (BH-2; Olympus, Tokyo, Japan). Images were obtained with a digital camera (MDS; Kodak, Rochester, NY) and processed using PhotoShop (Adobe, San Jose, CA). No subsequent alterations have been made to the images.

Immunocytochemistry

Deparaffinized and rehydrated pituitary and ovarian sections were incubated with 0.5% H_2O_2 for 30 min, treated with 0.5% Triton X-100 for 10 min, and blocked for 30 min at 25 C with blocking buffer B (3% goat serum and 2% blocking reagent in PBS). Sections were then incubated overnight at 4 C with either a 1:1000 dilution of rabbit antirecombinant seabream FSHβ serum or a 1:3000 dilution of rabbit anti-striped bass LHβ serum. Control sections were incubated with either preimmune sera or the respective antiserum preabsorbed with either 2 μg/ml native striped bass LHβ or 5 μg/ml seabream recombinant FSHβ. Peptide identity of seabream and striped bass LHβ is 93% (15), and the cross-reactivity of anti-striped bass LHβ peptide serum to seabream LHβ has been demonstrated (18). Excess antibody was removed by two 15-min washes in PBS. The Vectastain Elite ABC-peroxidase kit (Vector, Burlingame, CA) was used according to the manufacturer's instructions. In the preabsorbed antiserum assays, goat antirabbit IgG horseradish peroxidase conjugate (1:1000 dilution; Bio-Rad, Hercules, CA) was used instead of the Vectastain Elite ABC-peroxidase kit to reduce the background signal. Color development in sections was initiated with 3, 3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). After color development, sections were examined under a light microscope, and digital images were taken as described above.

Ovarian fragment incubation and GnRH analog treatments

The 1- to 2-mm-thick vitellogenic ovarian fragments obtained from 2- or 3-yr-old seabream were washed twice for 30 min with Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) containing 100 U penicillin and 100 μg streptomycin/ml and 25 mM HEPES (pH 7.4). In 24-well tissue plates, ovarian fragments (n = 4, about 400 mg each) were incubated in 1 ml fresh culture medium in a 20 C incubator with gentle shaking (60 rpm). GnRH agonist, mammalian GnRH analog (mGnRH-A)/[des-Gly10, D-Ala6, Pro(-Net)]-mammalian GnRH (Bachem, Torrance, CA), was added to the medium at concentrations of 0, 0.1, 1, 10, 100, and 1000 nM. After a 3-, 6-, or 18-h incubation, LH content in the incubation media was measured via ELISA, and transcript levels of *Fshβ*, *Lhβ*, and *Cgα* in the ovarian fragments were analyzed by quantitative PCR. To study the effect of GnRH antagonist on the secretion of ovarian LH, analog E [Ac-δ 3-Pro1, 4FD-Phe2, D-Trp3,6]-mammalian GnRH (Bachem) was added to the media (for 6 h incubation) at a concentration of 100 nM in either the presence or absence of 100 nM mGnRH-A.

LH ELISA

LH content in media was measured using a heterologous ELISA designed to specifically quantify the striped bass LH (19). This ELISA has been shown to work in a variety of perciform species, including the gilthead seabream (18). Unknown samples and standards were first preincubated with the primary antibodies (final dilution 1:150,000) in 2% normal goat serum and PBS containing 0.05% of Tween 20 at 4 C overnight and dispensed into 96-well plates precoated with striped bass LHβ for a 3-h incubation at 37 C. The antigen-antibody complexes were detected by addition of goat antirabbit-horseradish peroxidase and 3,3′, 5,5′-tetramethyl benzidine peroxidase substrate (KPL Inc., Gaithersburg, MD). The reaction was stopped with 1 M H_3PO_4. Absorbencies were read at 450 nm, using an automatic microplate reader (Thermomax; Molecular Devices, Sunnyvale, CA).

Statistical analyses

Data obtained via quantitative PCR on the transcript levels of *Fshβ*, *Lhβ*, and *Cgα* and ELISA on the levels of ovarian LH secretion were presented as the mean and SEM. Results from *in vitro* ovarian incubations as well as different tissues were examined using ANOVA followed by Duncan's multiple range test for the *Fshβ*, *Lhβ*, and *Cgα* transcript expression. The effects of GnRH analogs on the secretion of ovarian LH were analyzed using ANOVA followed by either Dunnett (control) test or Duncan's multiple range test. In all cases, significance was accepted at $P < 0.05$.

Results

Transcript expression of gonadotropin subunit genes in ovulated eggs and ovarian tissues

During the study of the ontogeny of the HPG axis in gilthead seabream, we discovered both *Lhβ* and *Fshβ* transcripts in ovulated eggs. Representative plots of the quantitative PCR amplification curves of *Fshβ* (Fig. 1A) and *Lhβ* (Fig. 1B) transcripts are shown. No significant amplification was found in the control samples (without reverse transcriptase in cDNA syntheses). To confirm this novel ovarian expression of gonadotropin subunit genes, we used additional approaches to investigate the expression of *Fshβ*, *Lhβ*, and *Cgα* transcripts in the vitellogenic ovary. Using RT-PCR, transcripts of *Fshβ* (Fig. 1C), *Lhβ* (Fig. 1D), and *Cgα* (Fig. 1E) were all found in ovary undergoing vitellogenesis. To compare transcript levels of *Fshβ* and *Lhβ* between ovaries and

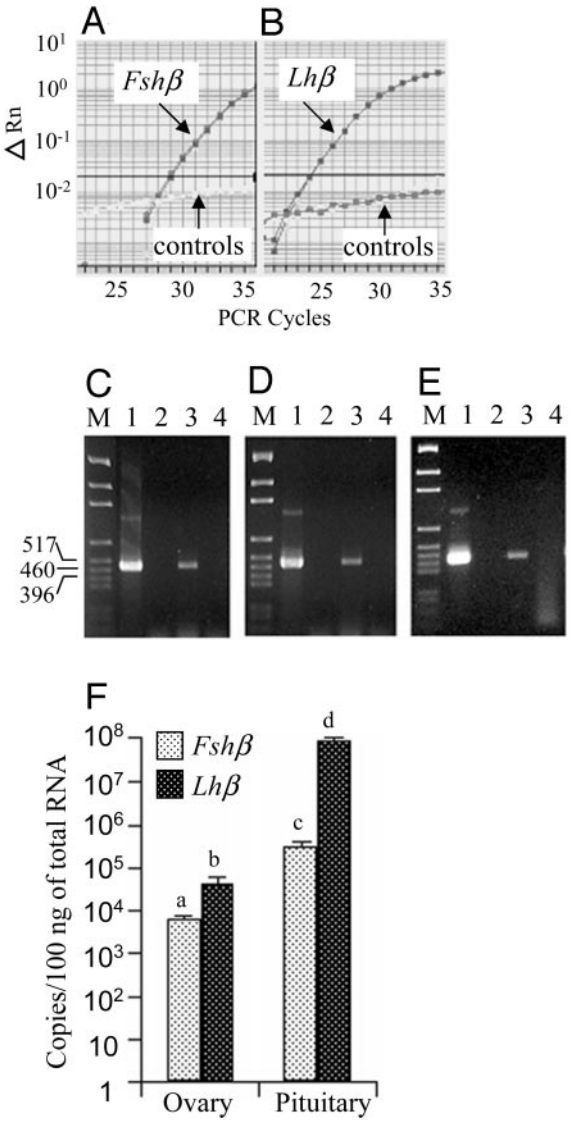

FIG. 1. The expression of *Fshβ*, *Lhβ*, and *Cgα* transcripts in ovulated eggs, pituitaries, and ovaries of gilthead seabream. Representative plots of quantitative PCR (triplicates) for measuring *Fshβ* transcript (A) and *Lhβ* transcript (B) in ovulated eggs; reverse transcriptase was omitted in controls. The expression of *Fshβ* (C), *Lhβ* (D), and *Cgα* (E) transcripts in vitellogenic ovary detected by RT-PCR. The transcript levels of *Fshβ* and *Lhβ* in the ovaries and pituitaries (from four females) measured by quantitative PCR (F). In C–E, 2 ng pituitary total RNA for lanes 1 and 2; 10 ng ovarian mRNA for lanes 3 and 4; lanes 2 and 4 are negative controls lacking reverse transcriptase in the reverse transcription reaction. M, DNA marker. In F, data (n = 4) are presented as the mean and SEM and analyzed for the difference in transcript levels using ANOVA followed by Duncan's multiple range test. Data points not sharing a *letter* (a, b, c, d) are significantly different.

pituitaries (from four females during the spawning season), *Fshβ* and *Lhβ* RNA standard curves were generated for use in quantitative PCR. Our data showed that levels of *Fshβ* transcripts, when presented as copies/100 ng total RNA, were about 6×10^3 in the ovary and 3×10^5 in the pituitary; levels of *Lhβ* transcripts were about 4×10^4 in the ovary and 8×10^7 in the pituitary (Fig. 1F).

Northern blot and sequence analyses of ovarian Fshβ and Lhβ and 5′-flanking region of Lhβ

To further investigate this novel expression of *Fshβ* and *Lhβ* in the ovarian tissues, Northern blot analyses were conducted. To detect the ovarian *Fshβ* and *Lhβ* transcripts and prevent the overexposed signals from pituitary *Fshβ* and *Lhβ* transcripts, much less pituitary RNA (2 μg of total RNA), compared with ovarian RNA (10 μg of mRNA), was used in this assay. Our results demonstrated that the transcript of *Fshβ* is about 0.6 kb in both pituitary and ovary (Fig. 2A), whereas the ovarian *Lhβ* transcript (about 1.1 kb), unexpectedly, is longer than the known pituitary *Lhβ* transcript (about 0.6 kb, Fig. 2B). Using RACE methodology, we isolated and sequenced the ovarian *Fshβ* and *Lhβ* cDNAs (Fig. 3 and supplemental Fig. 1). The homology between ovarian *Fshβ* cDNA and the published pituitary *Fshβ* cDNA (15) is 98% at the nucleotide level and 100% at the amino acid level (Fig. 3A and supplemental Fig. 1A). The ovarian *Lhβ* cDNA was found to contain the same open reading frame (ORF) but possess an additional 459 bases at the distal portion of the 5′-untranslated region, compared with the published pituitary *Lhβ* cDNA (Fig. 3B and supplemental Fig. 1B). Not considering the 459 bases at the distal portion of the 5′-untranslated region, the homology between ovarian *Lhβ* cDNA and the published pituitary *Lhβ* cDNA (15) is 97% at the nucleotide level and 99% at the amino acid level. We also PCR amplified the ORF region of *Lhβ* from pituitary cDNA using PLBF and PLBR primers (Table 1). Unlike the published pituitary *Lhβ* sequence, the ORF region of pituitary *Lhβ* that we obtained has 100% homology to the same region of the ovarian *Lhβ* cDNA (Fig. 3B and supplemental Fig. 1B).

In an attempt to investigate the tissue-specific expression of ovarian *Lhβ*, primers (LHBF1 and LHBR1) hybridized to the distal portion of the 5′-untranslated region of ovarian *Lhβ* were used for RT-PCR analysis. Our data demonstrated that ovarian *Lhβ* is not expressed in the pituitary. In addition, a longer amplicon (compared with the amplicon from ovarian cDNA) was generated when using genomic DNA as a positive control (Fig. 4A), indicating that additional ovarian intron(s) exist in the 5′ distal portion. Hence, we isolated the 5′-flanking region of *Lhβ* (GenBank accession no. AY606043). Sequence alignment and analyses revealed that ovarian *Lhβ*

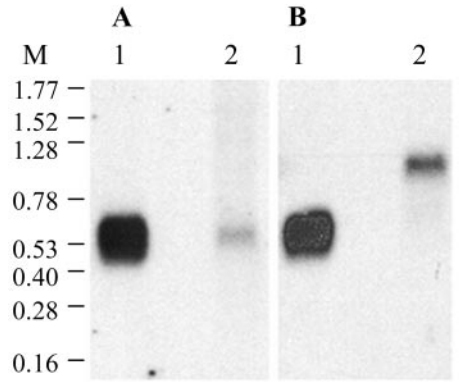

FIG. 2. Northern blot analyses of the pituitary and ovarian *Fshβ* (A) and *Lhβ* (B) transcripts. Lane 1, 2 μg pituitary total RNA; lane 2, 10 μg ovarian mRNA. M, RNA marker (kilobase).

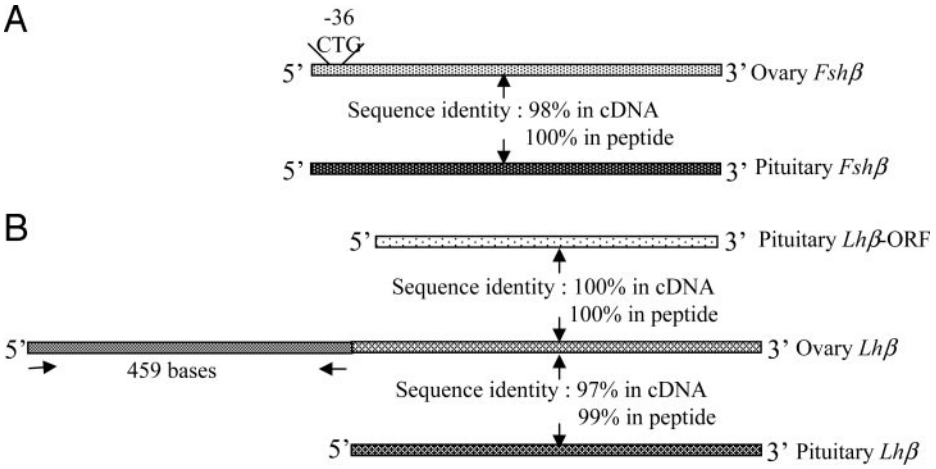

FIG. 3. Schematic summary of homology alignment and comparison of the seabream ovarian *Fshβ* (A) and *Lhβ* (B) cDNAs with the published pituitary *Fshβ* and *Lhβ* cDNAs and the sequence of pituitary *Lhβ* ORF (PCR product in this study). An alternative initiation codon CUG for *Fshβ* cDNA (A) is located 36 nucleotides upstream from predicted translation initiation site. cDNAs used in this comparison and the 5′ distal portion of ovarian *Lhβ* cDNA are distinctly labeled.

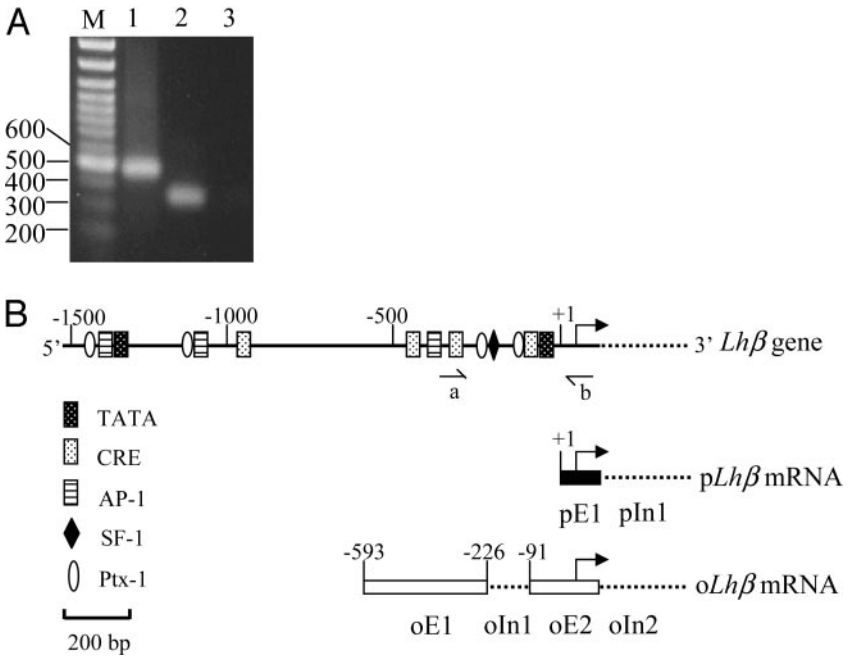

FIG. 4. Tissue-specific expression of ovarian *Lhβ* (A) and the structure and putative response elements of the *Lhβ* 5′-flanking region (B). The expression of ovarian *Lhβ* is found in the ovary but not pituitary (A, lanes 2 and 3) by RT-PCR using LHBF1 and LHBR1 primers; a longer amplicon is detected when using genomic DNA as template (lane 1). In A, 20 ng genomic DNA for lane 1, ovarian total RNA for lane 2, and pituitary total RNA for lane 3. M, DNA marker. In B, the *solid box* indicates the known pituitary (p) exon (E); the *open boxes* indicate the novel ovarian (o) exons; the *dashed lines* indicate introns (In). The *angled arrows* indicate the translation initiation sites. Putative TATA boxes and response elements including cAMP response element, activating protein 1, steroidogenic factor 1, and pituitary homeobox factor 1 are labeled with *distinctively filled shapes*. The relative positions are also labeled (+1 as the predicted transcription initiation site for pituitary *Lhβ*). a, LHBF1 primer; b, LHBR1 primer.

possesses the ovarian-specific exon 1, intron 1, and exon 2 and that the 3′-end portion of ovarian exon 2 is identical with pituitary exon 1 (Fig. 4B). The first TATA box is located 28 bp upstream of pituitary exon 1. No TATA box was found at the expected location of 25–35 bp upstream of ovarian exon 1. The other identified TATA box is located further upstream (−814 bp) from ovarian exon 1. Unlike pituitary *Lhβ*, ovarian *Lhβ* is driven by a TATA-less promoter. Several putative response elements such as steroidogenic factor 1, pituitary homeobox factor 1, activating protein 1, and cAMP response element were found in this 5′-flanking region (Fig. 4B).

Localization of the gonadotropin subunit gene expression

The SG phase of seabream folliculogenesis can be divided into three stages (20): SG-I (presence of yolk vesicles containing mucopolysaccharides in ooplasm), SG-II (presence of lipid droplets), and SG-III (presence of yolk granules). Using ISH, we detected the *Fshβ* transcript in the ooplasm of PG and SG-I oocytes (Fig. 5A); the *Lhβ* transcript in the ooplasm of SG-I, SG-II, and SG-III oocytes (Fig. 5, C and D); and the *Cgα* transcript in both PG and SG oocytes (Fig. 5F). No significant signal was seen in the control sections (hybridized with sense riboprobes; Fig. 5, B, E, and G).

Using immunocytochemistry, we detected the FSHβ, surprisingly, in the nuclei of PG and SG-I oocytes (Fig. 6A). No significant signal was seen in the preimmune serum controls (Fig. 6B). To further confirm the specificity of anti-FSHβ serum, preabsorbed antiserum controls were carried out on sections of pituitary and ovary. Signals of FSHβ were seen in the gonadotropes and PG oocytes (Fig. 6, C and E), whereas no significant signal was seen in preabsorbed antiserum controls (Fig. 6, D and F). The LHβ peptide was detected in the ooplasm of SG-II and SG-III oocytes (Fig. 6, G and H), and no significant signal was seen in the preimmune serum controls (Fig. 6I). The specificity of anti-LHβ serum was also confirmed using the preabsorbed antiserum on pituitary and ovarian sections. Signals of LHβ peptide were seen in the gonadotropes and SG-II oocytes (Fig. 6, J and L), whereas no

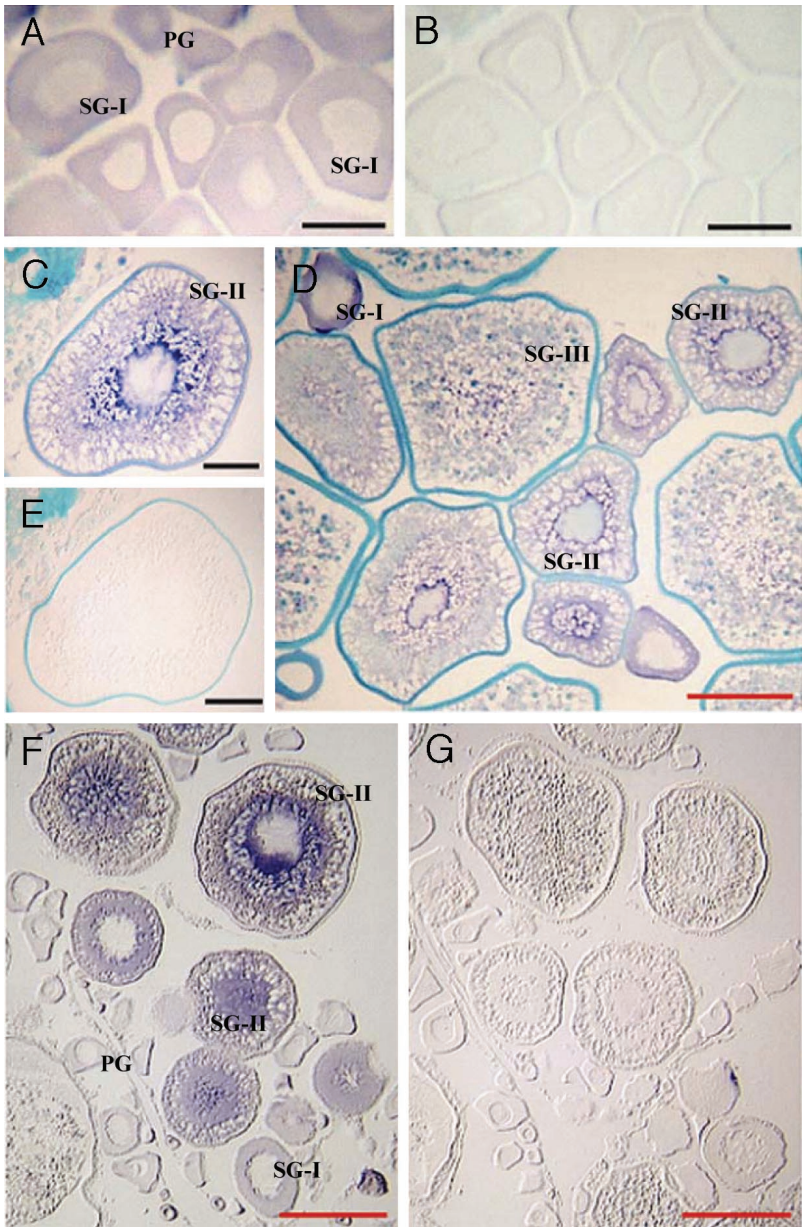

FIG. 5. Localization of *Fshβ*, *Lhβ*, and *Cgα* transcripts in seabream ovary. Photomicrographs of methyl green-counterstained sections (A and B) and fast green-counterstained sections (C–E). A distribution of *purple* signals for *Fshβ* in PG and SG-I oocytes (A) and *purple-blue* signals for *Lhβ* in SG-I, SG-II, and SG-III oocytes (C and D) are visible. The signals for *Cgα* in PG oocytes (*light purple*) and SG-I and SG-II oocytes (*purple-blue*) are also seen (F). No significant signal is apparent in control sections hybridized with sense riboprobe of *Fshβ* (B), *Lhβ* (E), or *Cgα* (G). *Scale*, 80 μm for *black bars*; 200 μm for *red bars*.

significant signal was seen in preabsorbed antiserum controls (Fig. 6, K and M).

Effects of GnRH analogs on the transcript expression of ovarian gonadotropin subunits and secretion of ovarian LH

Using the short-term *in vitro* ovarian fragment incubation, we demonstrated that mGnRH-A enhances the expression of ovarian *Fshβ*, *Lhβ*, and *Cgα*. Specifically, mGnRH-A significantly promotes *Fshβ* expression at 1, 10, 100, and 1000 nM (up to 2.7-fold) in a 3-h incubation at 100 nM (1.7-fold) in a 6-h incubation and at 100 and 1000 nM (up to 2.5-fold) in an 18-h incubation (Fig. 7A). The effects of mGnRH-A on the levels of ovarian *Lhβ* transcript were seen in a 3-h (at 1, 10, 100, and 1000 nM) and 6-h (at 100 nM) incubation. However, transcript levels did not increase more than 1.4-fold, and no significant change was seen in an 18-h incubation (Fig. 7B). mGnRH-A also enhances the ovarian *Cgα* expression at 0.1 nM (1.4-fold) in a 6-h incubation and at 0.1, 1, 100, and 1000 nM (up to 1.8-fold) in an 18-h incubation (Fig. 7C).

In terms of the secretion of ovarian LH, our results demonstrated that ovarian LH was spontaneously secreted and accumulated in the incubation media. LH levels in the media increased from undetectable at 0 h to about 1.4 ng/ml after 18 h of incubation (Fig. 8A, control). mGnRH-A (100 nM) significantly enhanced the secretion of ovarian LH (0.25 ng/ml in control *vs.* 0.56 ng/ml in mGnRH-A treatment) during a 6-h incubation (Fig. 8A). Moreover, data from three independent experiments showed that higher concentrations of mGnRH-A, 100 and 1000 nM in 6-h incubations, consistently and significantly ($P < 0.05$) enhanced the secretion of LH from ovarian fragments to the incubation media (from 0.8 to 1.8 ng/ml in Fig. 8B; one representative experiment is

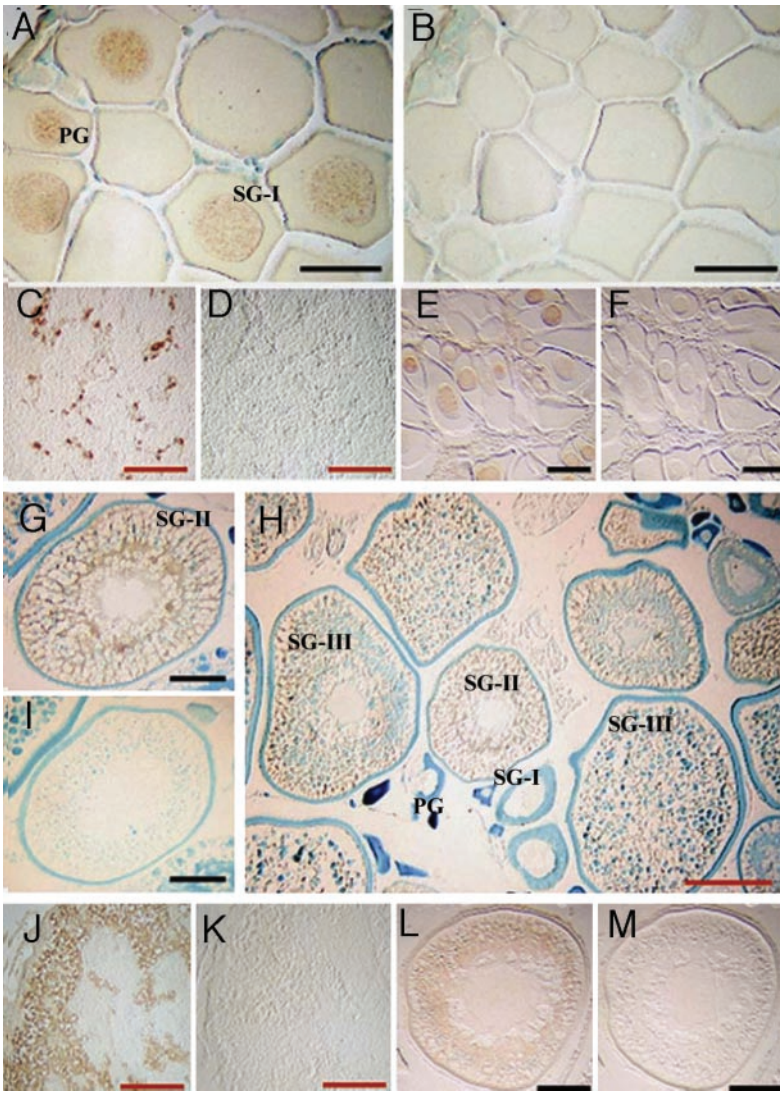

FIG. 6. Localization of FSHβ and LHβ peptides in seabream ovary. Photomicrographs of methyl green-counterstained sections (A and B) and fast green-counterstained sections (G–I). FSHβ peptides (*brown stain*) were detected in nuclei of PG and SG-I oocytes (A); no significant signal was found in preimmune control sections (B). In the study of antiserum specificity, FSHβ peptides were found in the pituitary gonadotropes (C) and PG oocytes (E); no significant signal was found in control (preabsorbed antiserum) pituitary (D) and ovarian (F) sections. LHβ peptides (*brown stain*) were seen in the ooplasm of SG-II and SG-III oocytes (G and H); no significant signal was found in the preimmune control sections (I). In the study of specificity of anti-LHβ serum, LHβ peptides were detected in the pituitary gonadotropes (J) and SG-II oocytes (L); no significant signal was found in control (preabsorbed antiserum) pituitary (K) and ovarian (M) sections. *Scale*: 80 μm for *black bars*; 200 μm for *red bars*.

shown). In addition, GnRH antagonist, analog E, at 100 nM, largely suppressed the spontaneous secretion ovarian LH, *i.e.* levels of ovarian LH in the media are below the detection limit (0.1 ng/ml) of our LH ELISA. However, the addition of 100 nM of mGnRH-A partially rescued the suppressive effect of analog E on LH secretion (Fig. 8C).

Discussion

The essential roles of FSH and LH, previously considered solely as pituitary hormones, in folliculogenesis have been well established (see introductory part of article). The novelty of our findings is that oocytes also expressed these well-characterized gonadotropin subunits that may serve as autocrine/paracrine factors participating in intraovarian communication. Our results showed that pituitaries expressed *Fsh*β and *Lh*β transcripts at much higher levels than ovaries when data were presented as copies per 100 ng total RNA (about 50-fold for *Fsh*β and 2000-fold for *Lh*β). However, in the fish used for this study, average ovary weight (76.5 g) was 7500-fold more than average pituitary weight (10.3 mg). The total production of *Fsh*β and *Lh*β transcripts in the ovary cannot be overlooked when accounting for the total tissue weight. Moreover, the seasonal fluctuation of plasma LH in seabream is between 1 and 15 ng/ml (18). Our *in vitro* results showed that the ovarian LH secretion to the media could reach 1.4 ng/ml after a 6-h incubation (see Fig. 8C, control). Considering both the transcription and secretion, production levels of ovarian gonadotropin subunits are considerable. In teleosts, much of the research on the involvement of LH in the control of oocyte maturation has focused on its role in promoting the production of MIH. The synthesis of MIH in granulosa cells (21) indicates that the LH receptivity of granulosa cells is the key step that triggers FOM and ovulation. The expression of *Lh*β in SG-III oocytes and ovulated eggs suggests that oocytes may directly participate, along with pituitary gonadotropins, in the regulation of FOM and ovulation through the action of ovarian LH. Our findings certainly alter the current understanding of the integration and coordination of both extraovarian and intraovarian signals, which ultimately determine the success of oocyte development.

Ovarian folliculogenesis is a complex process integrating

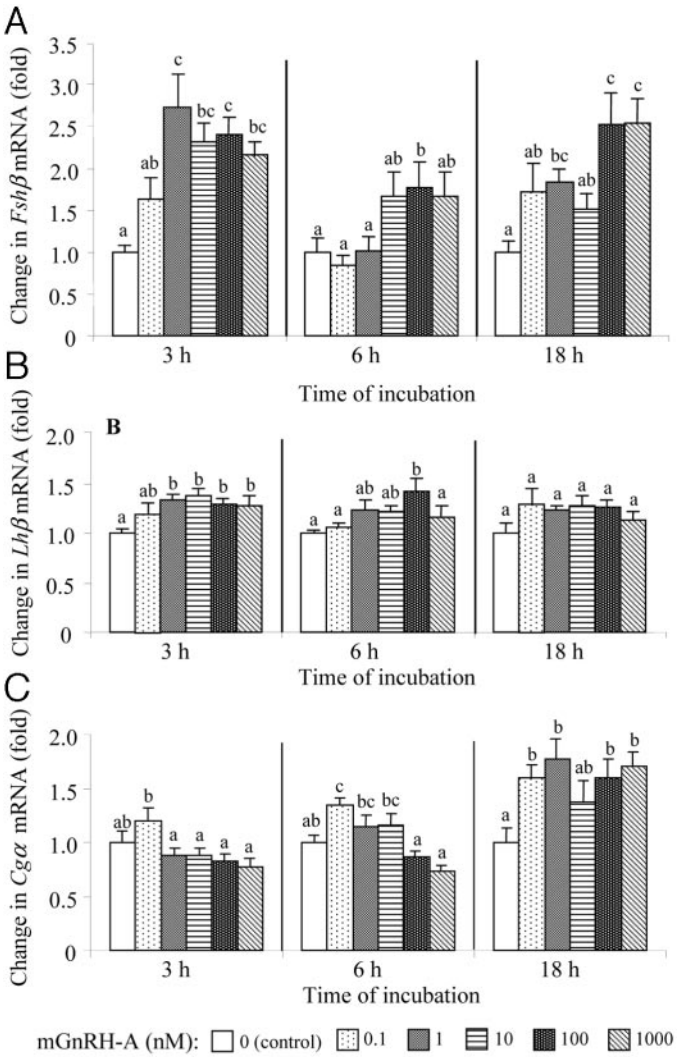

FIG. 7. GnRH agonist, mGnRH-A, enhances the expression of ovarian *Fshβ* (A), *Lhβ* (B), and *Cgα* (C) transcripts. Data (n = 4) were presented as the mean and SEM of fold difference, compared with controls. In each different period of incubation, one-way ANOVA followed by Duncan's multiple range test was performed to analyze the effect of mGnRH-A on the transcript expression. In all cases $P < 0.05$, with the exception of the last panel of B, the *Lhβ* expression after 18 h incubation. In each group, data points not sharing a *letter* (a, b, c) are significantly different.

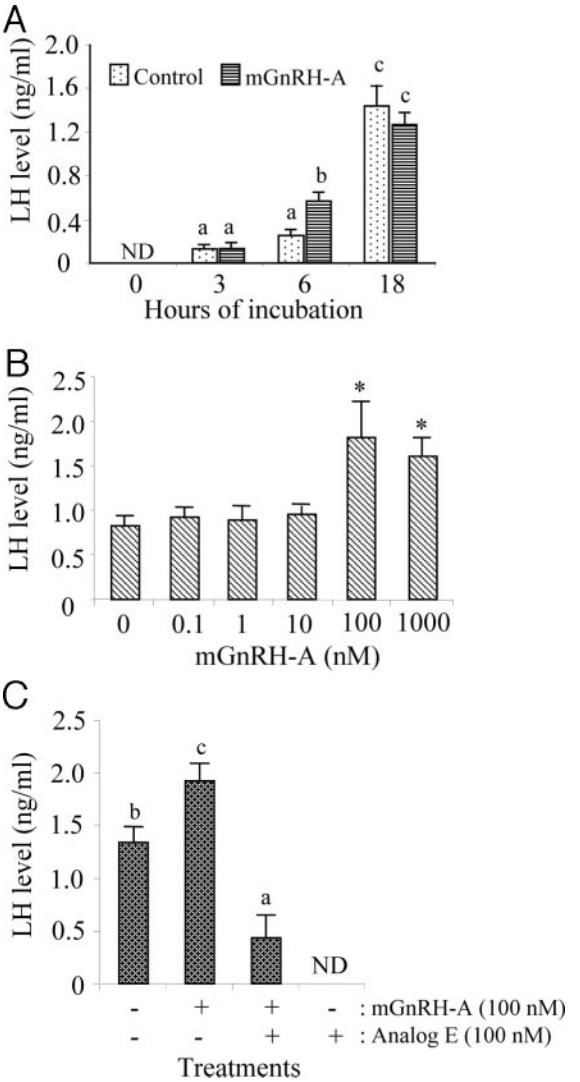

FIG. 8. Effects of GnRH agonist and antagonist on the secretion of ovarian LH. A time-course study using 100 *vs.* 0 nM (control) of mGnRH-A (A). One of three independent experiments at 6-h ovarian incubation with serial dilutions of mGnRH-A (B). The effect of GnRH antagonist, analog E, on the secretion of ovarian LH (C). Data (n = 4) were presented as the mean and SEM and analyzed using ANOVA for each data point followed by Duncan's multiple range test (A, C) and for each mGnRH-A concentration followed by Dunnett (control) test (B). Data points not sharing a *letter* (a, b, c) are significantly different (A, C). *Asterisk* represents a significant difference ($P < 0.05$) from controls (0 nM of mGnRH-A). ND, Not detected.

both systemic endocrine hormones (*e.g.* gonadotropins) and intraovarian factors. Known oocyte-derived factors are growth and differentiation factor-9 and bone morphogenetic protein-15. Additional factors are produced by follicle cells, such as steroids, IGFs, TGFα and -β, epidermal growth factor, fibroblast growth factor, inhibins, activins, and follistatins (22–24). Similarly in fish, the ovarian expression of IGF, epidermal growth factor, and activins, as well as their corresponding receptors, have been identified (25–28). The *in vitro* stimulation of oocyte maturation by these three intraovarian factors has also been demonstrated (29–31). These results indicate a certain similarity in the intraovarian communication between fish models and mammals. The two major ovarian follicle cell types, granulosa and thecal cells, not only mediate the gonadotropin signals from the endocrine system but also regulate the progression of folliculogenesis through intraovarian factors that signal oocytes. The expression of gonadotropin subunits in the oocytes suggests that ovarian gonadotropins may be involved in mediating the actions of the intraovarian factors. Thus, oocytes may use ovarian gonadotropins in response to the actions of intraovarian factors.

The discovery of ovarian FSHβ, LHβ, and *Cgα* has attracted our attention to the well-known gonadotropin regulator, GnRH, which has also been found in ovarian tissue but is not widely recognized as an intraovarian factor modulating folliculogenesis. The presence of GnRH-like peptides were first identified in luteinized rat ovaries (32), and ex-

pression of GnRH has been localized to granulosa cells of the follicles (33). The production of GnRH by specific cell types within the ovaries is common among most, if not all, vertebrate taxa, including gilthead seabream (34). Administration of GnRH analog to hypophysectomized female rats induced oocyte maturation and ovulation of mature follicles, which indicates the direct effect of GnRH on the gonad (35, 36). Furthermore, subsequent studies have confirmed the presence of GnRH receptor transcripts in the granulosa-luteal cells of human (37), granulosa cells of rat (38, 39), and oocytes of gilthead seabream (Kight, K.E., D. Alok and Y. Zohar, unpublished results and supplemental Fig. 2). The available data imply that endogenous ovarian GnRH may be involved in regulating the expression of ovarian gonadotropins through the GnRH receptor present on either the oocytes or the granulosa cells (in which case secondary messengers may reach the oocytes via gap junctions).

For more than two decades, the short-term *in vitro* ovarian fragment incubation protocol has been established and applied in fish models to study the production of gonadal steroids and their regulation by other hormones (40). Using the same methodology, our results demonstrated that GnRH agonist promotes the expression of *Fshβ*, *Lhβ*, and *Cgα* transcripts. In cultured pituitary cells of tilapia, mGnRH-A led to an increase of *Lhβ* (3- to 4-fold) and *Fshβ* (1.7-fold) transcripts (41). Unlike in cultured pituitary cells, a relatively low mGnRH-A response (only up to 1.4-fold increase) was seen for ovarian *Lhβ* transcript expression. In contrast to ovarian *Lhβ*, high mGnRH-A response (up to 2.7-fold increase) was seen for ovarian *Fshβ* transcript expression. A discernible mGnRH-A response was also seen for ovarian *Cgα* transcript expression in an 18-h incubation. Results from Northern blot and cDNA sequence analyses indicate that both ovarian FSHβ and LHβ are encoded by the genes that encode the pituitary FSHβ and LHβ, respectively. The difference is that ovarian *Lhβ* is driven by a different promoter (a TATA-less promoter) upstream of the pituitary *Lhβ* promoter (a canonical TATA promoter), which suggests that the transcriptional machinery and control of ovarian *Lhβ* may differ from that of pituitary *Lhβ*. Thus, relative to each other, these two promoters may use a different subset of transcription activators. This may, in part, be the reason for the low responsiveness of ovarian *Lhβ* expression to mGnRH-A treatment.

Our results also indicate that ovarian LH may mediate the action of intraovarian GnRH. In the absence of exogenous GnRH, *in vitro* incubated ovarian fragments secrete LH spontaneously, and the level of LH in the media increased with the length of the incubation (see Fig. 8, control groups). This spontaneous secretion is largely suppressed in the presence of a GnRH antagonist (analog E, see Fig. 8C). Similarly, analog E was shown to suppress GnRH-induced gonadotropin secretion from goldfish pituitaries (42). The suppressive effect of analog E on ovarian LH secretion indicates that this GnRH antagonist blocks the action of endogenous ovarian GnRH on LH secretion from the ovary, suggesting an endogenous ovarian GnRH-LH axis in the gilthead seabream. This suggestion is further supported by the fact that exogenous mGnRH-A partially rescues the suppressive effect of analog E on LH secretion from the ovarian fragments (see Fig. 8C). This endogenous GnRH-induced LH secretion from the ovary may also be partially responsible for the lack of an exogenous mGnRH-A induction effect on ovarian LH secretion when studying low dosages of mGnRH-A (less than 100 nM) or longer incubation times (18 h).

In light of the unexpected finding of FSHβ in the oocytes' nuclei, which has been further confirmed using preabsorbed antiserum, the manner in which nuclear FSHβ influences the cellular function of oocytes should be investigated and established. FSHβ has not been reported as a transcription factor or protein that translocates to the nucleus. However, studies have revealed that fibroblast growth factors can be diverted from a cytoplasmic secretory pathway to the nuclear targeting pathway using an in-frame upstream CUG as an initiation codon (43). The action of such a nuclear-targeting growth factor has also been correlated to ribosomal gene transcription (44). In the seabream *Fshβ* cDNA, we also noted an in-frame CUG codon 36 nucleotides upstream from the predicted translation initiation site (see Fig. 3A and supplemental Fig. 1A). Therefore, it is possible that FSHβ may use the same mechanism for its nuclear targeting. Furthermore, the presence of functional FSH receptors in oocytes has been demonstrated (45), which raises the possibility of autocrine action of ovarian FSH in oocyte development.

Presently there is no immunoassay available to quantify FSHβ peptide levels or detect Cgα peptide in perciform fish, including gilthead seabream. Consequently, the cell localization of Cgα and the secretion of ovarian FSH and its regulation cannot be further studied without the development of additional tools. As noted earlier, a number of intraovarian factors have been identified as coregulators of folliculogenesis. Gonadal inhibins and activins were first described as regulators of pituitary function, specifically for the regulation of FSHβ expression (46–49). Activin receptors (whose activities are possibly blocked by inhibins) have been found on oocytes (50). It is possible that these peptides may regulate oocyte development by modulating the expression and production of ovarian FSHβ and LHβ.

Although the expression of gonadotropin subunit genes in testis have been reported, *Fshβ* and *Cgα* in mouse testis (51) and *Lhβ* and *Cgα* in rat testis (52), the deduced peptides in rat testicular *Lhβ* cDNAs, were either truncated or initiated differently from pituitary *Lhβ* (53). Our results demonstrate, for the first time in vertebrates, that FSHβ and LHβ are synthesized *de novo* in oocytes. Moreover, they also provide evidence for the presence of a GnRH-gonadotropin axis in the ovary. Our findings add to the growing body of literature on intraovarian communication in vertebrates. Collectively, this information supports the intriguing possibility that the ovarian GnRH-gonadotropin axis may be involved in the bidirectional communication between oocytes and their companion somatic cells during oocyte development. In gilthead seabream, the discovery of the local ovarian GnRH-gonadotropin axis, in addition to the systemic HPG axis, reveals a new level of complexity in the integration and coordination of the endocrine, paracrine, and autocrine systems, which use some of the same chemical signals during oocyte development. Confirmation of these results in other vertebrate models, including mammals, will certainly strengthen the value of this discovery.

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