General and Comparative Endocrinology 233 (2016) 8–15

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Production and characterization of monoclonal antibodies against recombinant tethered follicle-stimulating hormone from Japanese eel *Anguilla japonica*

Dae-Jung Kim [a,1], Chae-Won Park [b,1], Dong-Wan Kim [b], Hong-Kyu Park [b], Munkhzaya Byambaragchaa [b], Nam-Sil Lee [a], Sun-Mee Hong [c], Mi-Young Seo [a], Myung-Hwa Kang [d], Kwan-Sik Min [b,*]

[a] Aquaculture Research Division, National Institute of Fisheries Science (NIFS), Busan 46083, Republic of Korea
[b] Animal Biotechnology, Graduate School of Future Convergence Technology, Institute of Genetic Engineering, Hankyong National University, Anseong 17579, Republic of Korea
[c] Dept. of Research and Development, Institute of Gyeongbuk Marine Bioindustry, Ulgin 36315, Republic of Korea
[d] Department of Food Science & Nutrition, Hoseo University, Asan 31499, Republic of Korea

ARTICLE INFO

Article history:
Received 26 November 2015
Revised 26 April 2016
Accepted 28 April 2016
Available online 9 May 2016

Keywords:
Japanese eel
Follicle-stimulating hormone
Monoclonal antibody
Recombinant

ABSTRACT

We prepared monoclonal antibodies (mAbs) against a recombinant tethered follicle-stimulating hormone (rec-FSH) from Japanese eel *Anguilla japonica* that was produced in *Escherichia coli*. Positive hybridomas (clones eFA-C5, eFA-C10, eFA-C11, eFA-C12, eFA-C13, and eFB-C14) were selected by using the eel FSH antigen in ELISA, and anti-eel FSH mAbs were purified from culture supernatants by performing affinity chromatography. Three of the 6 mAbs were characterized and their isotypes were identified as IgG2b (eFA-C5 and eFA-C11) and IgG1 (eFB-C14). In western blotting assays, the mAbs recognized the antigen as a 24.3-kDa band, and further detected bands of 34 and 32 kDa in the supernatants of CHO cells transfected with cDNA encoding tethered eel FSHβ/α and LHβ/α, respectively. PNase F-mediated deglycosylation of the recombinant proteins resulted in a drastic reduction in their molecular weight, to 7–9 kDa. The mAbs eFA-C5 and eFA-C11 recognized the eel FSHα-subunit that is commonly encoded among glycoprotein hormones, whereas eFB-C14 recognized the eel FSHβ-subunit, and immunohistochemical analysis revealed that the staining by these mAbs was specifically localized in the eel pituitary. We also established an ELISA system for detecting rec-tethered FSHβ/α and LHβ/α produced from CHO cell lines. Measurement of biological activities *in vitro* revealed that only weak activity of rec-FSHβ/α was detected. The activity of rec-LHβ/α was found to be increased in a dose-dependent manner for eel oocyte maturation.

1. Introduction

The gonadotropins (GTHs) follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are produced in the pituitary gland of all vertebrates, including fish species, and they are key regulators of gonadal development and reproduction (Aizen et al., 2012). Members of this glycoprotein family, which includes FSH, LH, chorionic gonadotropin (CG), and thyroid stimulating hormone (TSH), are composed of noncovalently linked α- and β-subunits (Min et al., 2004). Whereas the α-subunits are common to these glycoproteins, the β-subunits differ among them (Min et al., 1996).

In fish, as in other vertebrates, reproductive activity is regulated by the hypothalamus-pituitary-gonad axis and 2 types of GTH (FSH and LH) secreted from the pituitary gland (Swanson et al., 2003). The cDNAs encoding FSHβ-subunit and LHβ-subunit from Japanese eel *Anguilla japonica* have been cloned, and the changes in GTH transcripts during artificial maturation of the eels have been studied extensively (Nagae et al., 1996, 1997; Saito et al., 2003; Yoshiura et al., 1999).

Japanese eel is one of the most important fish species being aquacultured in East Asian countries, including Japan and Korea (Kagawa et al., 1998). Japanese eels caught from the wild possess immature gonads, and further gametogenesis is arrested under captive conditions (Kazeto et al., 2008). However, gonadal development can be induced by administering gonadotropic reagents such as salmon pituitary extract (SPE) (Kim et al., 2007b; Kazeto et al., 2008; Kobayashi et al., 2010).

* Corresponding author.
E-mail address: ksmin@hknu.ac.kr (K.-S. Min).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ygcen.2016.04.030
0016-6480/

Female eels are sexually immature under normal cultivation conditions, but most of these eels complete vitellogenesis after repeated injections of SPE (Kim et al., 2007b; Kagawa et al., 2013). SPE treatment alone does not completely induced oocyte maturation and ovulation. In recent work, implantation of a single osmotic pump loaded with SPE was demonstrated to induce vitellogenesis and increase the gonadosomatic index (GSI) (Kagawa et al., 2013), the implantation of an osmotic pump loaded with human CG (hCG) was shown to induce spermatogenesis and increase the GSI values at 35–42 days post-implantation (Kagawa et al., 2009). Moreover, in *in vitro* experiments, oocytes were also shown to acquire the ability to respond to maturation-inducing steroid (MIS) at the migratory-nucleus stage (>700 mm in diameter) (Kim et al., 2007b; Kagawa et al., 2013).

Recombinant (rec) eel FSH and LH have recently been widely reported to exhibit biological activity in investigations on reproductive endocrinology when produced from baculovirus in silkworm larvae (Kobayashi et al., 2010), *Drosophila* S2 cells (Kazeto et al., 2008), and *Pichia pastoris* (Kamei et al., 2003; Ohta et al., 2007). However, no study has reported complete induction of artificial spawning by hormonal treatments. Previously, we also reported the production of rec-glycoproteins including equine CG (eCG), eFSH, human FSH (hFSH), human erythropoietin (hEPO), dimeric-hEPO and human thrombopoietin (hTPO) in CHO-K1 cells, and found that these recombinant derivatives exhibited biological activity both *in vitro* and *in vivo* (Jeong et al., 2010; Kim et al., 2007a; Min et al., 1996, 2004; Naidansuren and Min, 2009, 2010; Park et al., 2009; Saneyoshi et al., 2001).

In this study, we produced specific monoclonal antibodies that can be used to detect eel FSHα- and β-subunits. The monoclonal antibodies against rec-tethered eel FSHβ/α protein produced in *Escherichia coli* recognized those rec-tethered proteins (FSHβ/α and LHβ/α) obtained from CHO cells using by using western blotting analysis and immunohistochemistry for eel pituitary. We also analyzed the biological activities by measuring the percentages of GVBD *in vitro*.

2. Materials and methods

2.1. Materials

The cloning vector pCR2.1 and expression vector pcDNA3 were purchased from Invitrogen (Carlsbad, CA, USA). Restriction enzymes and a DNA ligation kit were purchased from Takara (Tokyo, Japan). PRO-PREP protein extraction solution was obtained from Intron Biotechnology (Seoul, Korea). A Lumi-Light Western Blot kit was bought from Roche (Basel, Switzerland). Horse serum, goat serum, an avidin–biotin-HRP (ABC) detection kit, 3,30-diaminobenzidine (DAB), hematoxylin and methyl green were purchased from Vector Laboratories (Burlingame, CA, USA) anti-mouse secondary antibody was purchased from Dako Cytomation (Glostrup, Denmark). Competent *E. coli* cells were from Yeastern Biotech Co. Ltd. (Seoul, Korea). Serum-free CHO-S-SFM II and Lipofectamine 2000 reagents were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT, USA). Oligonucleotides were synthesized by Genotech (Daejeon, Korea). All other chemicals were obtained from local suppliers.

2.2. Tissues

Cultivated Japanese eels were purchased from a commercial dealer and transferred to recirculating seawater tanks maintained at 20 °C. Eel pituitaries were collected from >3 eels (400–500 g) after they were anesthetized using ethyl aminobenzoate, and then stored at −80 °C until use. All experiments were conducted according to the Guidelines for the Care and Use of Animals, Hankyong National University and National Institute of Fisheries Science.

2.3. Preparation of cDNAs encoding eel α-subunit and FSHβ- and LHβ-subunits

Total RNA was extracted from eel pituitary tissues stored at −80 °C by using the TRIzol reagent (Invitrogen) and cDNA was synthesized by using the SuperScript First-Strand Synthesis System according to the manufacturer's instructions. The primers for eel α-subunit (sense: 5′-ACC ATG ATG GTG TGT CCA GGA-3′, antisense: 5′-TAA AAT TTG TGG GGT AGT AGC AGG TGC-3′) were designed based on the nucleotide sequence (Nagae et al., 1996). The primers for eel FSHβ-subunit (sense: 5′-ACC ATG CAT CTG GCT GTC ACA GCG CTG-3′, antisense: 5′-CTA GTG GGT CAG ACA GCC-3′) and eel LHβ-subunit (sense: 5′-ACC ATG TCA GTC TAC CCA GAA -3′, antisense: 5′-CTA CGC GGG GAG GCT GGC CCG CTG-3′) were synthesized based on the previously reported nucleotide sequence of the cloned gene (Nagae et al., 1997; Yoshiura et al., 1999). Next, PCR was performed using 2.5 U of *Pfu* polymerase in a Quick Thermo-II PCR machine, and the amplification protocol included 30 cycles of denaturation (91 °C for 1 min), annealing (37 °C for 1 min), and extension (72 °C for 2 min). PCR products were analyzed using electrophoresis performed in Tris-acetate buffer containing EDTA. The PCR products were ligated into the pGEMT vector and the sequence of the entire region of the cDNAs was verified using automated DNA sequencing as previously reported (Min et al., 2004).

2.4. Construction of vectors for rec-tethered eel FSHβ/α and LHβ/α

Construction of cDNAs encoding the rec-tethered eel proteins was modified as previously reported (Min et al., 2004). A cDNA encoding full-length eel FSHβ-subunit (signal sequence, 22 amino acids; mature protein, 105 amino acids) was fused with the 5′ end of the eel α-subunit cDNA, which included the coding sequence of the mature protein (93 amino acids) but lacked the signal sequence. The same method was also used to fuse the tethered eel LHβ/α cDNA (signal sequence, 24 amino acids; mature protein, 116 amino acids), with the 5′-end of the eel α-subunit cDNA.

The following primers were used in constructing rec-tethered eel FSHβ/α: Primer 1, T-eel FSHβ-F (5′-GAA TTC ATG CAT CTG GCT GTC ACA GCG-3′); Primer 2, T-eel FSHβ-R (5′-GTT GTT GGG ATA CTG GGT CAG ACA GCC TGA-3′), which contains the first 4 codons of the mature α-subunit and the last 5 C-terminal codons of FSHβ-subunit; Primer 3, T-eel α-F (5′-TGT CTG ACC CAC TAT CCC AAC AAC GAA ATG-3′), which contains the sequence corresponding to the last 4 C-terminal codons of eel FSHβ-subunit and the first 6 codons of the α-subunit from which the signal sequence was removed; and Primer 4, T-eel α-R (5′-TAC GTC GAC TTA AAA TTT GTG GTA GTA GCA-3′). Next, we constructed rec-tethered eel LHβ/α by using these primers: Primer 1, T-eel LHβ-F (5′-GAA TTC ATG TCA GTC TAC CCA GAA TGC-3′); Primer 2, T-eel LHβ-R (5′-GTT GTT GGG ATA CGC GGG GAG GCT GGC CCG-3′), as the T-eel FSHβ-R primer; Primer 3, T-eel α-F (5′-AGC CTC CCC GCG TAT CCC AAC AAC GAA ATG-3′); and Primer 4, T-eel α-R, the same primer as that used for T-eel FSHβ/α.

The first PCR was performed using primer sets 1–2 to 3–4. Fragments were annealed and subjected to the second PCR by using Primers 1 and 4 to generate rec-tethered eel FSHβ/α and LHβ/α, as shown in Fig. 1. These fragments were digested with *Eco* RI/*Sal* I and ligated into the same sites of pcDNA3.1 expression vector (designated as pcDNA3-T-eel-FSHβ/α and pcDNA-T-eel-LHβ/α).

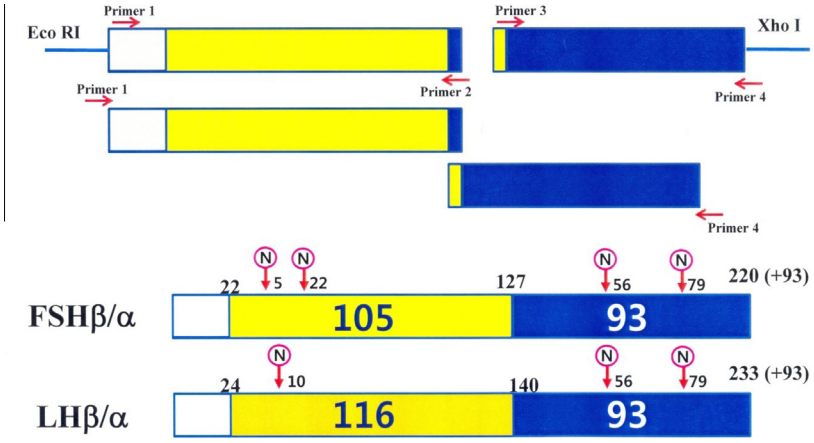

Fig. 1. Construction of rec-tethered eel FSHβ/α and LHβ/α. Construction of rec-tethered eel FSHβ/α and LHβ/α by using overlapping PCR and an expression transfer vector. The first PCR was performed using Primer pairs 1–2 and 3–4 (Step 1). The fragments were subjected to the second PCR by using Primers 1 and 4 to generate tethered-eCG. These fragments were digested with *Eco*RI/*Xho*I and ligated into the same sites of pcDNA3, which generated pcDNA3-T-eel-FSHβ/α and pcDNA3-T-eel-LHβ/α. The circled "N" denotes N-linked oligosaccharides; eel FSHβ-subunit contains 2N-linked glycosylation sites, whereas eel LHβ-subunit contains only one.

2.5. Production of rec-tethered eel FSHβ/α in E.coli

The antibodies were manufactured according to a previously reported method used in our laboratory (Naidansuren and Min, 2012). The rec-tethered eel FSHβ/α cDNA was amplified using a forward primer that, contained an *Nde* I site in the mature protein region of FSHβ-subunit lacking the signal sequence (5′-AGT CAT ATG TGT GGT CTC GCC AAC-3′) and a reverse primer including Xho I site (5′-CTC GAG TTA AAA TTT GTG GTA GTA GCA-3′). The rec-tethered eel FSHβ/α cDNA was cut at the *Nde* I and *Xho* I sites and then subcloned into the pRSET expression vector to construct a pRSET vector containing the rec-tethered eel FSHβ/α full-length cDNA, and this was used for transformation into *E. coli*. Three colonies were selected for producing the recombinant protein, and after preparing large-volume cultures, the protein was purified by using Ni-NTA Sepharose column chromatography with 250 mM imidazole.

2.6. Immunization procedure

Two BALB/c mice (6–8 weeks old) were immunized with the purified rec-tethered eel FSHβ/α. Briefly, each mouse was immunized 5 times with 50 μg of the purified protein once every 2 weeks; the first immunization was performed using complete Freund's adjuvant, and incomplete Freund's adjuvant was used for subsequent immunizations. One week after the last immunization, blood was collected by making a vertical incision in the tail vein, and then antibody titers were determined using ELISA. Lastly, 3 days before the cell fusion, 20 μg of rec-tethered eel FSHβ/α (without any adjuvant) was injected intravenously as previously reported (Bayat et al., 2013).

2.7. ELISA

Mouse serum titrations and screening of hybridoma supernatants were performed using ELISA. Plates were coated with 50 μL of rec-tethered eel FSHβ/α (10 μg/mL) dissolved in Phosphate buffered saline (PBS), incubated at room temperature (RT) for 1 h, then incubated overnight incubation at 4 °C. Subsequently, the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) for 3 min and blocked with 2.5% bovine serum albumin (BSA) for 1 h at RT. After washing the wells 3 times, mouse sera (in 2-fold serial dilutions starting from 1:500) were added and incubated for 1 h, following which the wells were again washed with PBS-T. Next, horseradish peroxidase-conjugated rabbit anti-mouse Ig antibodies (1:1000) were added to the wells and incubated for 1 h at RT, and after washing, 50 μL of tetramethylbenzidine (TMB) substrate was added to each well and the plates were incubated at room temperature in the dark. After 15 min, the reaction was terminated by adding 15 μL of a stop solution (20% H_2SO_4) to each well, and the optical density (OD) of the reactions was measured at 450 nm by using an ELISA reader (BioTek, Winooski, VT, USA). The mouse that showed the higher antibody titer was selected for the fusion. To screen the antibody production of hybridoma cells, the same method was applied on the cell supernatants.

2.8. Hybridoma cell production

Briefly, mouse myeloma Sp2/0 cells, used as fusion partners, were cultured and propagated in RPMI-1640 culture medium (Gibco) containing 10% FBS. Spleen cells from the immunized mouse were mixed with the Sp2/0 cells at a ratio of 1:5. The mixture was washed twice with pre-warmed RMPI-1640 (37 °C), and then pre-warmed 50% polyethylene glycol 1500 was used for the fusion. Selective hypoxanthine-aminopterin-thymidine medium was then used for selecting hybridoma cells, and the reactivity of the culture supernatants was tested using ELISA. Lastly, positive hybridomas were cloned using a limiting dilution process (Bayat et al., 2013).

2.9. Antibody purification and isotype determination

Anti-eel FSHβ/α monoclonal antibodies were purified from culture supernatants by means of affinity chromatography performed using a Hi-Trap Protein G column (GE Healthcare, Uppsala, Sweden). Briefly, culture supernatants were filtered through 0.45-μm filters and their pH was adjusted to 7.5, and captured antibodies were eluted from the column by using 0.1 M glycine-HCl pH 2.7. The eluted antibodies were dialyzed against PBS at pH 7.5, and the reactivity of the purified antibodies was determined using the ELISA method. Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (all diluted 1:1000) were coated in the wells of ELISA plates, and supernatants of the growing hybridomas were added to each well. The isotype was then determined according to the method described in the "ELISA" subsection.

2.10. Cells and transfection of pcDNA3 T-eel-FSHβ/α and pcDNA3 T-eel-LHβ/α vectors

CHO-KI cells were cultured in Ham's F12 growth medium containing penicillin (50 U/mL), streptomycin (50 mg/mL), glutamine (2 mM), and 10% FCS and incubated at 37 °C under 5% CO_2. Cultured CHO-K1 cells were transfected with the expression vectors by using the liposome transfection method as described previously (Min et al., 2004). After 4–6 h of transfection, 250 μL of 20% FBS was added to the wells, and the cells were maintained at 37 °C in a CO_2 incubator for 24 h. On the following day, transfected cells were washed twice, and then 500 μL of serum-free medium was added and the cells were incubated at 37 °C for 48 h.

Next, 6–8 clones of stably transfected pools were selected based on growth in G-418 containing medium. After incubation of the selected stable cells in 20 mL of CHO-S-SFM-II at 37 °C for 48 h, the culture media were collected and centrifuged at 15,000 rpm at 4 °C for 20 min to remove the cell debris.

2.11. Western blotting analysis and deglycosylation of rec-tethered FSHβ/α and LHβ/α produced in CHO-K1 cells

Briefly, samples were centrifuged at 105,000×*g* at 4 °C–8 °C for 5 min, and the supernatants were transferred to fresh 1.5-mL tubes. Protein concentration was measured using the Bradford method (Bradford, 1976). Rec-tethered eel FSHβ/α and LHβ/α were processed to remove the added glycans by using an N-deglycosylation enzyme. To remove all N-linked glycans, the rec-tethered samples were incubated for 24 h at 37 °C with PNase F [2 μL of enzyme (2.5 U/mL)/30 μL of samples + 8 μL of 5 × reaction buffer]. The reaction was stopped by boiling for 10 min.

Samples were separated using SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes by using a semi-dry electroblotter apparatus. After blotting, the membranes were blocked with a 5% blocking reagent for 1 h and then incubated for 1 h with 1:1500 dilutions of the monoclonal antibodies against eel FSH. The membranes were washed to remove unbound antibody, and then incubated for 30 min with secondary HRP-linked anti-mouse IgG antibodies (1:2000). Lastly, the membranes were incubated for 5 min with 2 mL of Lumi-Light substrate solution, and after discarding the solution, the membranes were exposed to X-ray films for 1–10 min.

2.12. Immunohistochemistry of eel pituitary glands

Immunohistochemical (IHC) staining of eel pituitary samples (GSI 1.2%) was performed using the Vectastain ABC kit according to the method described previously (Kim et al., 2011). The samples were fixed in 70% DEPC-treated water Ethanol at 4 °C for 24 h. The fixed samples were then rehydrated using a graded ethanol series (twice 10 min each in 100%, 95%, and 70%) and embedded in paraffin. Paraffin-embedded tissues were sectioned into 5-μm slices on a microtome, and the sections were mounted onto poly-L-lysine-coated slides. The slides were boiled in 10 mM sodium citrate for 10 min and chilled on ice for 20 min, and then washed in 3% hydrogen peroxide for 10 min, and blocked with 5% horse serum for 1 h at RT. After washing, the slides were incubated overnight at 4 °C with the primary antibodies eFA-C5, eFA-C11, and eFB-C14 (1:500) diluted in 5% horse serum blocking buffer. The slides were then incubated with a biotinylated secondary antibody (polyclonal swine anti-mouse IgG, 1:1000). Tissue sections were immunostained using the ABC detection kit and stained with DAB.

2.13. Detection of rec-tethered eel FSHβ/α and LHβ/α by using sandwich ELISA

To establish an ELISA detection system for rec-tethered eel FSHβ/α and LHβ/α, eFA-C5, eFA-C11 and eFB-C14 (200 ng/100 μL/well) were coated in 96-well plates and then incubated overnight at 4 °C. After blocking with 0.5% casein in PBS for 1 h at 37 °C, the plates were washed 3 times with PBS-T. Next, dilution samples of antigen (0–800 ng/mL) and an undiluted solution of rec-FSHβ/α or rec-LHβ/α produced from CHO-K1 cells were added and incubated for 1 h at 37 °C. After washing 3 times with PBS-T, HRP-conjugated anti-eel monoclonal antibodies (eFA-C5, eFA-C11, and eFB-C14) diluted 100-fold in PBS were added by intersection and incubated for 1 h at room temperature. After washing, TMB was added as substrate and the test was continued as described in the section "ELISA."

2.14. In vitro oocyte-maturation assay

Biological activities of rec-tethered eel FSHβ/α and LHβ/α were examined using an *in vitro* oocyte-maturation assay. Cultured female Japanese eels (approximately 300–500 g) were obtained from a commercial dealer (Yangman Fishery Cooperation, Cheonnam, Korea). To induce sexual maturation, the eels were intraperitoneally injected with salmon pituitary extract (SPE, 30 mg/kg body weight) once a week. After 10 injections, ovarian fragments containing oocytes at the migratory-nucleus stage (850–900 μm in diameter) were collected through an incision in the abdomen and then stored in ice-cold culture medium (pH 7.4, L-15) supplemented with 10 mM HEPES, 100 mg/L streptomycin, and 70 mg/L penicillin. We placed roughly 20–30 oocytes in each well of culture plates and preincubated them for 2 h and then added various doses of rec-FSHβ/α and -LHβ/α (0.01, 0.1, and 1 μg/mL), SPE (1.0 mg/mL), or 17,20β-dihydroxy-4-pregnen-3-one (DHP, 100 ng/mL). After incubating the oocytes in media for 24 h at 20 °C, the percentage of oocyte maturation was calculated by counting the oocytes that underwent germinal vesicle breakdown (GVBD). GVBD was identified based on observing the presence or absence of the germinal vesicle by using a binocular microscope. Experimental procedures were performed under the guidelines of the animal care committee of National Institute of Fisheries Science.

3. Results

3.1. Production of the antigen FSHβ/α in E.coli

The pRSET vector was transfected into *E. coli*, and 3 colonies were selected for producing the rec-tethered protein. After culture, SDS-PAGE analysis was performed on the whole-cell, soluble, and inclusion-body fractions of the 3 strains. The rec-tethered eel FSHβ/α protein was detected in all strains, and the protein was present at the highest level in the whole cells of Strain 3. Thus, we purified the Strain 3 sample by performing 1st Ni-NTA Sepharose column chromatography with 250 mM imidazole (see Fig. 1B in Ref [#]) as previously reported (Naidansuren et al., 2011; Naidansuren and Min, 2012). The rec-tethered eel FSHβ/α protein produced in *E. coli* was 24.3 kDa in molecular weight.

3.2. Production and characterization of monoclonal antibodies against rec-tethered eel FSHβ/α

After 2 mice were immunized with rec-tethered eel FSH β/α, the splenocytes of the higher-titer mouse were fused with Sp2/0 cells. The supernatants of the growing hybridoma cells were screened based on reactivity by using 96-well ELISA plates coated with dif-

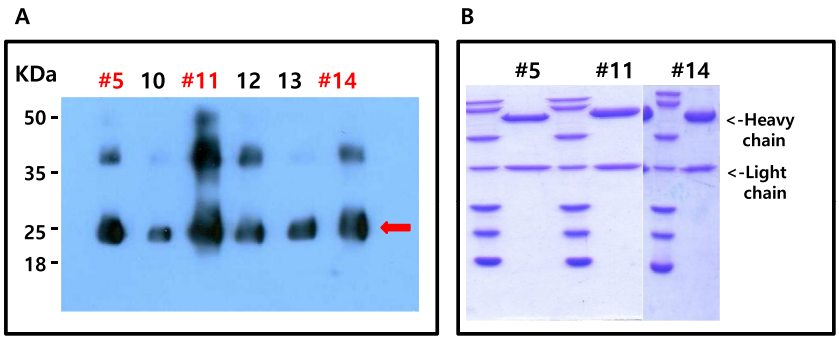

Fig. 2. Western blotting assay and characterization for monoclonal antibodies. (A) Western blotting assay performed on selected antibodies. The antigen (50 ng) was subjected to SDS-PAGE, blotted, and then detected with each antibody. The 2nd antibody used was goat anti-mouse (1:3000). Strong band signals were obtained with antibodies #5, #11, and #14. B) Characterization of antibodies. Antibody isotypes were identified as IgG2b (#5 and #11) and IgG1 (#14).

ferent concentrations of rec-tethered eel FSHβ/α (1000, 500, 250, 125, 62, 31, 15, and 0 ng/mL) (data not shown). In this screening assay, we selected 6 positive clones (#5, #10, #11, #12, #13, and #14), and then we also analyzed a serial dilution of the antibody in the case of #14 (see Fig. 2 in Ref [#]). We tested the binding between the antibodies and the antigen (loading: 50 ng) by performing SDS-PAGE/western blotting; the analysis performed using the antibodies showed the presence of a band of about 24 kDa (Fig. 2A). The antibodies were purified using a Hi-Trap Protein G column, and their isotypes were identified as IgG2b (#5 and #11) and IgG1 (#14) (Fig. 2B).

3.3. Western blotting of rec-tethered eel FSHβ/α and LHβ/α produced from CHO-K1 cells

We next examined the ability of the monoclonal antibodies to recognize the rec-tethered proteins in western blots performed on the supernatants and lysates of cells transfected with the expression vectors pcDNA3-T-eel-FSHβ/α and pcDNA-T-eel-LHβ/α. The results presented in Fig. 3A show that the antibodies specifically recognized a 34-kDa band (FSHβ/α) and a 32-kDa (LHβ/α). The antibodies #5 and #11 interacted with both FSHβ/α and LHβ/α, whereas the antibody #14 only interacted with FSH β/α.

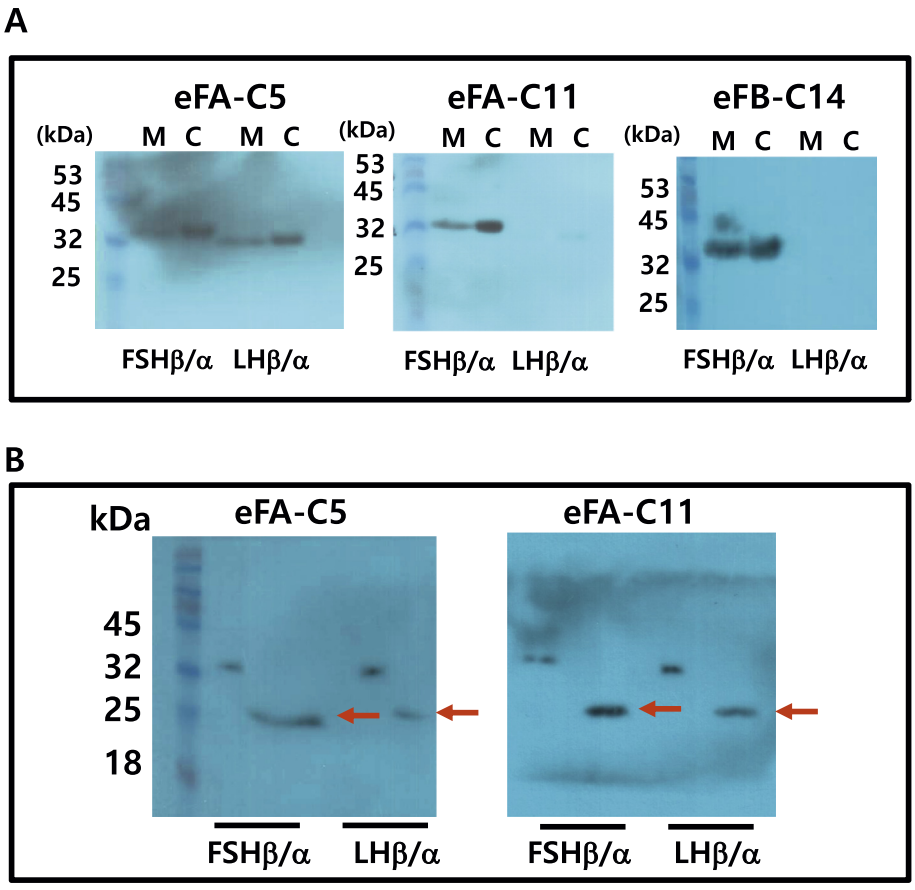

Fig. 3. Western blotting analysis of rec-tethered eel FSHβ/α and LHβ/α produced in CHO cells. (A) In western blots, the antibodies specifically recognize rec-tethered eel FSHβ/α and LHβ/α proteins produced in CHO cells. The supernatants of cells transfected with pcDNA3-T-eel-FSHβ/α and pcDNA3-T-eel-LHβ/α were collected and electrophoresed on SDS-PAGE gels and then transferred to PVDF membranes for immunoblotting. (B) Western blotting results obtained after N-linked deglycosylation treatment. Rec-tethered eel FSHβ/α and LHβ/α were digested with the N-linked deglycosylation enzyme and separated on a 12% SDS-PAGE gel. Antibody binding was visualized as described in Section 2. The results of a representative experiment are shown. Red arrows indicate the bands with reduced molecular weight obtained after PNase F treatment. M: medium; C: cell lysate; #5, #11, and #14: antibody numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

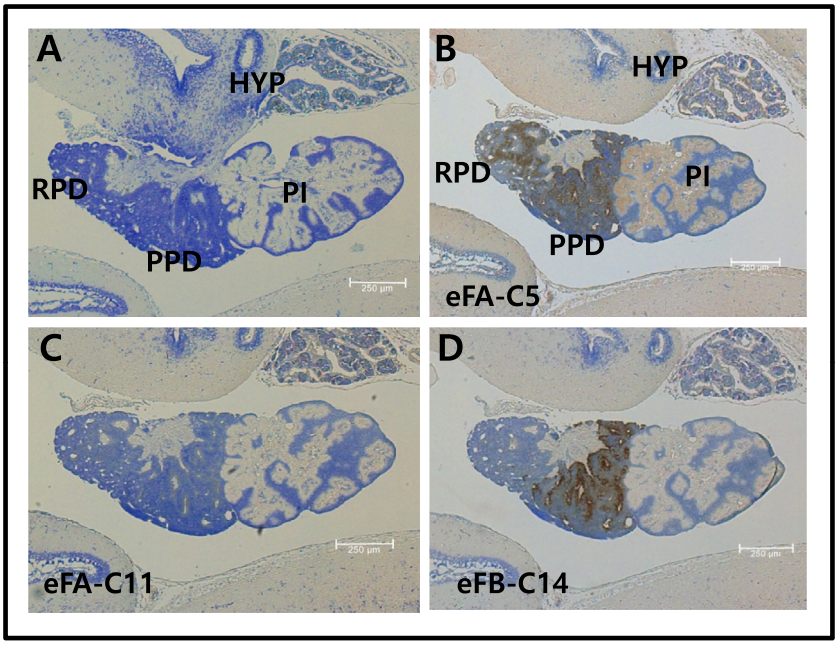

Fig. 4. Localization of eel α-subunit and FSHβ-subunit expression in the immature feminized eel pituitary. Adjacent sections of the pituitary were stained with antibodies, which specifically binds to FSHα-subunit (eFA-C5 and eFA-C11) and FSHβ-subunit (eFA-C14). (A) Preimmune serum (1:1000) was used as a negative control. (B–D) The specific site around the PPD in the pituitary was stained with the 3 monoclonal antibodies (eFA-C5, eFA-C11, and eFB-C14; 1:500) and detected with secondary antibodies. Representative IHC analyses was shown and swine anti-mouse IgG secondary antibodies (1:1000). Scale bars, 250 μm. RPD: rostral pars distalis; PPD: proximal pars distalis; PI: pars intermedia; HYP: hypothalamus.

Thus, we conclude that 2 of the 3 antibodies (#5 and #11) interact with α-subunit and the other antibody (#14) binds to FSHβ-subunit; we designated these monoclonal antibodies as eFA-C5, eFA-C11, and eFB-C14, respectively.

In order to analyze the glycosylation state of the rec-tethered proteins, we performed western blotting on samples that were treated with PNase F to remove all N-linked glycans. Our results (Fig. 3B) showed that the molecular weight of the deglycosylated protein was markedly decreased, to 7–9 kDa. These results suggest that the deglycosylated proteins were recognized by the monoclonal antibodies produced in this study.

3.4. Immunohistochemistry of eel pituitary

To determine the cell types that are responsible for eel α-subunit and FSHβ-subunit protein expression in the pituitary, we performed IHC analysis on sections of immature eel pituitary. Eel α-subunit antibody (eFA-C5) was strongly localized in the proximal pars distalis (PPD) and rostral pars distalis (RPD) (Fig. 4B), but eFA-C11 antibody was weakly localized in the same site (Fig. 4C). The detection of eel FSHβ-subunit (eFB-C14) indicated that this protein is also strongly expressed in the PPD of the eel pituitary (Fig. 4D). Thus the antibodies generated in this study could be used in IHC analysis to identify the cell types that express the subunits of the glycoprotein hormone FSH in the eel pituitary.

3.5. Development of an ELISA system for detecting rec-tethered eel FSHβ/α and LHβ/α

To establish an ELISA system for rec-tethered eel FSHβ/α, the 3 antibodies were coated in 96-well plates (as "capture" antibodies), and then different dilutions of the antigen (1–800 μg/mL) were coated as a standard. After rec-FSHβ/α and LHβ/α were added to the plates, HRP-labeled antibodies eFA-C5, eFA-C11, and eFB-C14 were added by intersection (as "detection" antibodies) (see Fig. 3A in Ref [#]). In this study, we determined from the standard curve that the optimal result was obtained with the combination between eFA-C5 antibody coating and eFA-C11 HRP labeling (data not shown). Furthermore, we also performed ELISA by using dilutions of the HRP-labeled antibody eFA-C11. These results suggested that the 100–3200-fold dilution range of the HRP-labeled antibody efficiently described the standard curve (0–400 ng/mL) (see Fig. 3B in Ref [#]). We also performed ELISA by using rec-tethered eel LH as standard (0–400 ng/mL). These results suggested that ELISA analysis is almost the same curve ($y = 0.006x$ for FSH; $y = 0.001x + 0.072$ for LH). Lastly, we analyzed the rec-tethered eel FSHβ/α and LHβ/α proteins from the CHO-K1 cell line; the quantities of these 2 rec-tethered proteins obtained were 76.7 and 39.7 ng/mL, respectively. The quantities of rec-FSHβ/α produced from stable cell lines were also analyzed by these ELISA method (see Fig. 3C in Ref [#]).

3.6. In vitro oocyte maturation

Measurement of the biological activities of rec-FSHβ/α *in vitro* revealed that in the rec-FSHβ/α treatment, the percentages of GVBD were increased slightly (10%) at the dose of 1 μg/mL. By contrast, the GVBD percentages after rec-LHβ/α treatment were increased significantly ($P < 0.05$) in a dose-dependent manner: 15% at 0.1 μg/mL and 30% at 1 μg/mL. The GVBD percentages of the SPE- and DHP-treatment groups were 35% and 100%, respectively (Fig. 5).

4. Discussion

We raised 3 monoclonal antibodies (eFA-C5, eFA-C11, and eFB-C14) against a recombinant protein corresponding to residues 1–220 of eel FSHβ/α. The availability of these monoclonal antibodies allowed us, for the first time, to analysis rec-tethered eel FSH. Western blotting analysis, which can be used to detect mature proteins, revealed a protein of the same molecular weight as the rec-protein in the cultured cells. Furthermore, we used those antibodies in IHC

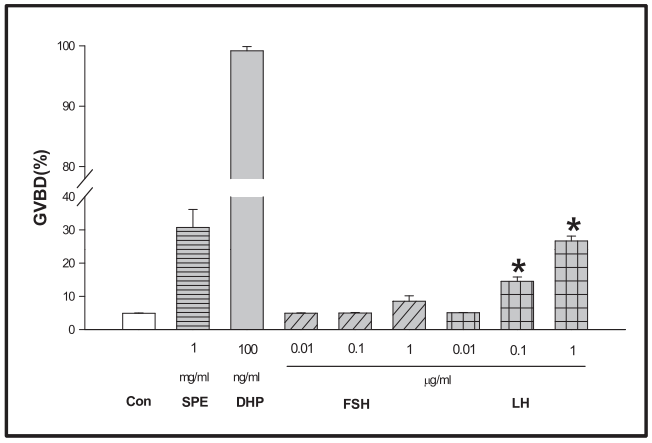

Fig. 5. Effects of rec-eel FSHβ/α and LHβ/α on germinal vesicle breakdown (GVBD) in oocytes of Japanese eel *in vitro*. SPE, salmon pituitary extract; DHP, 17,20β-dihydroxy-4-pregnen-3-one. Each value represents the mean and SEM of results from three replicates. Immunohistochemical staining was described in Section 2. *Significant difference from the groups ($P < 0.05$).

analysis of the eel pituitary, and we also established an ELISA system for detecting eel FSHβ/α and LHβ/α produced from CHO cells.

In this study, high-affinity monoclonal antibodies against eel FSH were produced and characterized: eFA-C5 and eFA-C11 recognized a specific band corresponding to eel α-subunit, whereas eFA-C14 bound to FSHβ-subunit. Thus, we detected rec-eel FSHβ/α and LHβ/α with eFA-C5 and eFA-C11 antibodies at around 34 and 32 kDa, respectively, in western blotting analysis.

Previously, antisera against eel GTHα, FSHβ, and LHβ were produced in rabbit by immunizing with synthetic peptides (Kamei et al., 2003). A broad band at 26 kDa and multiple bands above 50 kDa were detected using the anti-GTHα-subunit antiserum. In the yeast *P. pastoris*, 2 bands at 16.4 and 26.4 kDa were detected using the anti-FSHβ-subunit antiserum. In protein extracts prepared from eel pituitaries, an approximately 30–40-kDa molecular mass protein was detected with anti-eel FSHβ-subunit antiserum. The molecular mass of purified eel FSH was found to be approximately 33 kDa (Kamei et al., 2005). The rec-GTHα-subunit and LHβ-subunit produced using *Drosophila* S2 cells were single peptides with estimated molecular masses of 17.5 and 16.5 kDa, respectively, whereas the rec-FSHβ-subunit was detected as 2 bands (15.5 and 14 kDa) (Kazeto et al., 2008). In the baculovirus system used in silkworm larvae, rec-FSH and rec-LH were identified as specific bands corresponding to a molecular size of approximately 30–35 kDa (Kobayashi et al., 2010). Our results closely were agree with the results of previous studies showing that rec-FSH and rec-LH were detected at about 30–35 kDa in mammalian cells. In our previous studies, we have also produced recombinant mammalian FSHs by using mammalian cell lines expressing equine FSH (Saneyoshi et al., 2001) and bovine FSH (Min et al., 2003) and transgenic mice expressing human FSH (Kim et al., 2007c).

In this study, deglycosylation performed using PNase F drastically reduced the protein molecular weight, to 7–9 kDa. The molecular weight of rec-eel FSH in yeast was also previously shown to be decreased following deglycosylation by N-glycosylase F (Kamei et al., 2003); moreover, after treatment with N-glycopeptidase F, α-subunit and FSHβ-subunit were detected as 13 and 15 kDa proteins, respectively (Kamei et al., 2005). In the case of trout FSH and LH produced from baculovirus, the molecular mass of these 2 rec-hormones was decreased to 5–6 kDa (Ko et al., 2007). These results suggest that rec-eel FSH and LH are glycosylated in mammalian cells and that their molecular weight is decreased following PNase F treatment to about 5–9 kDa in the cell types in which they are expressed. Thus, oligosaccharides protect the circulating glycohormones against clearance *in vivo* (Naidansuren and Min, 2009, 2010). The data obtained using this approach suggest that the tethered mature FSHβ/α and LHβ/α are 34 and 32 kDa glycoproteins, respectively. This small difference in molecular weight was caused by glycosylation. Whereas only one N-linked glycosylation site is present in LHβ-subunit, FSHβ-subunit contains 2N-linked glycosylation sites.

In teleosts, GTHs are produced in the PPD, and FSHβ-subunit was localized in the PPD at the vitellogenic stage (Ozaki et al., 2007). Immunoreactive FSH cells were detected in the PPD, but not in other parts of these immature eel pituitary sections (Kamei et al., 2005). Moreover, according to the previous work, LH cells were not observed in the immature eel pituitary. We also determined here that the expression of FSHα-subunit and FSHβ-subunit was localized in the PPD. In this study, we established an ELISA system by using monoclonal antibodies against eel FSH, and we report here that this ELISA system could be effectively used for detecting rec-tethered eel FSHβ/α and LHβ/α proteins produced in mammalian cells.

The biological activities of rec-FSHβ/α and rec-LHβ/α were confirmed *in vitro*. Only weak activity of rec-FSHβ/α was detected in the oocyte-maturation assay, but the activity of LHβ/α was found to be increased in a dose-dependent manner. These results do not agree with the previous finding that rec-FSHβ/α expressed using baculovirus in silkworm larvae showed higher potency than LHβ/α in the induction of oocyte maturation *in vitro* (Kobayashi et al., 2010). The differences in the mean GSI measured with rec-FSH and rec-LH produced by *Drosophila* S2 cells were not statistically significant *in vivo* (Kazeto et al., 2008). The disparity in the results is probably due to the differences in glycosylation stoichiometry. Thus, determining the activities of rec-FSH would be essential for further elucidation of the specific biological function in eel oocyte maturation.

In summary, the results presented here clearly show that we have obtained specific monoclonal antibodies against the eel α-subunit and FSHβ-subunit. Our data suggest that the antibodies could be analyze the quantity of rec-eel FSH and LH hormones.

Acknowledgments

This work was supported by the project 'Seeding production of eel, *Anguilla japonica* by artificial induction of sexual maturation' (R2016003) of the NIFS, Korea, and the Export Strategical Item (D11503115H480000110) of KIMST, Korea.

References

Aizen, J., Kobayashi, M., Selicharova, I., Sohn, Y.C., Yoshizaki, G., Levavi-Sivan, B., 2012. Steroidogenic response of carp ovaries to piscine FSH and LH depends on the reproductive phase. Gen. Comp. Endocrinol. 178, 28–36.

Bayat, A.A., Yeganeh, O., Ghods, R., Zarmani, A.H., Arderani, R.B., Mahmoudi, A.R., Mahmoudian, J., Haghighat-Noutash, F., Jeddi-Tehrani, M., 2013. Production and characterization of a murine monoclonal antibody against human ferritin. Avicenna J. Med. Biotechnol. 5, 212–219.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Jeong, Y.H., Yoon, J.T., Min, K.S., 2010. Biological functions of the COOH-terminal amino acids of the a-subunit of tethered equine chorionic gonadotropin. Reprod. Dev. Biol. 34, 47–53.

Kagawa, H., Iinuma, N., Tanaka, H., Ohta, H., Okuzawa, K., 1998. Effects of rearing period in seawater on induced maturation in female Japanese eel *Anguilla japonica*. Fish Sci. 64, 77–82.

Kagawa, H., Horiuchi, Y., Kasuga, Y., Kishi, T., 2009. Oocyte hydration in the Japanese eel (*Anquilla japonica*) during meiosis resumption and ovulation. J. Exp. Zool. A Ecol. Genet. Physiol. 311, 752–762.

Kagawa, H., Sakurai, Y., Horiuchi, R., Kazeto, Y., Gen, K., Imaizumi, H., Masuda, Y., 2013. Mechanism of oocyte maturation and ovulation and its application to seed production in the Japanese eel. Fish Physiol. Biochem. 39, 13–17.

Kamei, H., Ohira, T., Yoshiura, Y., Uchida, N., Nagasawa, H., Aida, K., 2003. Expression of a biologically active recombinant follicle stimulating hormone of Japanese eel *Anguilla japonica* using methylotropic yeast, Pichia pastoris. Gen. Comp. Endocrinol. 134, 244–254.

Kamei, H., Kawazoe, I., Kaneko, T., Aida, K., 2005. Purification of follicle-stimulating hormone from immature Japanese eel, *Anguilla japonica*, and its biochemical properties and steroidogenic activities. Gen. Comp. Endocrinol. 143, 257–266.

Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (fsh) and luteinizing hormone (lh): production of biologically active recombinant fsh and lh by Drosophila S2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938–946.

Kim, B.S., Naidansuren, P., Min, K.S., 2007a. Biological activity of recombinant human thrombopoietin. J. Life Sci. 17, 1497–1505.

Kim, D.J., Bae, J.Y., Kim, E.O., 2007b. Changes in sex steroid hormones and ovarian development during artificial maturation of female eel, *Anguilla japonica*. Integ. Biosci. 11, 117–124.

Kim, M.O., Kim, S.H., Lee, S.R., Shin, M.J., Min, K.S., Lee, D.B., Lee, S.W., Kim, K.S., Kim, S.J., Ryoo, Z.Y., 2007c. Ectopic expression of tethered human follicle-stimulating hormone (hFSH) gene in transgenic mice. Transgenic Res. 16, 65–75.

Kim, D.J., Kawazoe, I., Jung, J.H., An, C.M., Kim, Y.C., Aida, K., 2011. Purification and characterization of luteinizing hormone from pituitary glands of rockfish, *Sebastes schlegeli*. Comp. Biochem. Physiol. 160, 104–109.

Ko, H., Park, W., Kim, D.J., Kobayashi, M., Sohn, Y.C., 2007. Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies. J. Mol. Endocrinol. 38, 99–111.

Kobayashi, M., Hayakawa, Y., Park, W., Banba, A., Yoshizaki, G., Kumamaru, K., Kagawa, H., Nagaya, H., Sohn, Y.C., 2010. Production of recombinant Japanese eel gonadotropins by baculovirus in silkworm larvae. Gen. Comp. Endocrinol. 167, 379–386.

Min, K.S., Hattori, N., Aikawa, J., Shiota, K., Ogawa, T., 1996. Site-directed mutagenesis of recombinant equine chorionic gonadotropin/luteinizing hormone: differential role of oligosaccharides in luteinizing hormone- and follicle-stimulating hormone-like activities. Endocrine J. 43, 585–593.

Min, K.S., Kang, M.H., Yoon, J.T., Jin, H.J., Seong, H.H., Chang, Y.M., Chung, H.J., Oh, S.J., Yun, S.G., Chang, W.K., 2003. Production of biological active single chain bovine LH and FSH. AJAS 16, 498–503.

Min, K.S., Hiyama, T., Seong, H.W., Hattori, N., Tanaka, S., Shiota, K., 2004. Biological activities of tethered equine chorionic gonadotropin (eCG) and its deglycosylated mutants. J. Reprod. Dev. 50, 297–304.

Nagae, M., Todo, T., General, K., Kato, Y., Young, G., Adachi, S., Yamauchi, K., 1996. Molecular cloning of the cDNAs encoding pituitary glycoprotein hormone α- and gonadotropin IIβ-subunits of the Japanese eel, *Anguilla japonica*, and increase of their mRNAs during ovarian development induced by injection of chum salmon pituitary homogenate. J. Mol. Endocrinol. 16, 171–181.

Nagae, M., Adachi, S., Yamauchi, K., 1997. Changes in transcription of pituitary glycoprotein α- and gonadotropin IIβ-subunits during ovarian development induced by repeated injections of salmon pituitary homogenate in the Japanese eel, *Anguilla japonica*. Fish Physiol. Biochem. 17, 179–186.

Naidansuren, J., Min, K.S., 2009. Development and characterization of hyperglycosylated recombinant human erythropoietin (HGEPO). Reprod. Dev. Biol. 33, 77–83.

Naidansuren, J., Min, K.S., 2010. Biological activity of human dimeric hyperglycosylated erythropoietin (dHGEPO) fusion proteins. Reprod. Dev. Biol. 34, 289–297.

Naidansuren, P., Min, K.S., 2012. Polyclonal antibody to a 37-kDa recombinant protein derived from bovine 20α-hydroxysteroid dehydrogenase. Reprod. Dev. Biol. 36, 109–114.

Naidansuren, P., Park, C.W., Kim, S.H., Nanjidsuren, T., Park, J.J., Yun, S.J., Sim, B.W., Hwang, S., Kang, M.H., Ryu, B.Y., Hwang, S.Y., Yoon, J.T., Yamanouchi, K., Min, K. S., 2011. Molecular characterization of bovine placental and ovarian 20α-hydroxysteriod dehydrogenase. Reproduction 142, 723–731.

Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. Biol. Reprod. 77, 970–977.

Ozaki, Y., Ishida, K., Saito, K., Ura, K., Adachi, S., Yamauchi, K., 2007. Immunohistochemical changes in production of pituitary hormones during artificial maturation of female Japanese eel *Anguilla japonica*. Fish Sci. 73, 574–584.

Park, J.J., Naidansuren, J., Yoon, J.T., Min, K.S., 2009. Function of the tethered rec-eCG in rat and equine receptors. Reprod. Dev. Biol. 33, 229–236.

Saito, K., Lokman, P.M., Young, G., Ozaki, Y., Matsubara, H., Okumura, H., Kazeto, Y., Yoshiura, Y., Aida, K., Adachi, S., Yamauchi, K., 2003. Follicle-stimulating hormone β, luteinizing hormone β and glycoprotein hormone a subunit mRNA levels in artificially maturation Japanese eel *Anguilla japonica* and naturally maturing New Zealand longfinned eel *Anguilla dieffenbachia*. Fish Sci. 69, 146–153.

Saneyoshi, T., Min, K.S., Ma, X.J., Nambo, Y., Hiyama, T., Tanaka, S., Shiota, K., 2001. Equine follicle-stimulating hormone: molecular cloning of β-subunit and biological role of the asparagine-linked oligosaccharide at asparagine[56] of α-subunit. Biol. Reprod. 65, 1686–1690.

Swanson, P., Dickey, J.T., Campbell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53–59.

Yoshiura, Y., Suetake, H., Aida, K., 1999. Duality of gonadotropin in a primitive teleost, Japanese eel (*Anguilla japonica*). Gen. Comp. Endocrinol. 114, 121–131.