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Posted Date: 16 May 2024

doi: 10.20944/preprints202405.1089.v1

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## Article

# Stable Production of a Recombinant Eel Luteinizing Hormone Analog with High Potency in CHO DG44 Cells

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**Abstract:** We produced a recombinant eel luteinizing hormone (rec-eel LH) analog with high potency in Chinese hamster ovary DG44 (CHO DG44) cells. The eel LH mutant (LH-M), which had a linker comprising the equine chorionic gonadotropin (eCG)  $\beta$ -subunit carboxyl-terminal peptide (CTP) region (amino acids 115 to 149), was inserted between the  $\beta$ -subunit and  $\alpha$ -subunit of wild-type tethered eel LH (LH-wt). Monoclonal cells transfected with the eel LH-wt and eel LH-M plasmids were isolated from five and nine clones of CHO DG44 cells, respectively. The secreted quantities abruptly increased on day 3, with peak levels of 5,000–7,500 ng/mL on day 9. The molecular weight of eel LH-wt was 32–36 kDa while that of eel LH-M increased to approximately 38–44 kDa, indicating the detection of two bands. Treatment with peptide N-glycanase F decreased the molecular weight by approximately 8 kDa. The oligosaccharides at the eCG  $\beta$ -subunit O-linked glycosylation sites were appropriately modified post-translation. The EC<sub>50</sub> value and maximal responsiveness of eel LH-M increased by approximately 2.90- and 1.29-fold, respectively, indicating that the mutant exhibited more potent biological activity than eel LH-wt. Phosphorylated extracellular regulated kinase (pERK1/2) activation resulted in a sharp peak 5 min after agonist treatment, with a rapid decrease thereafter. These results indicate that the new rec-eel LH analog which contains the eCG  $\beta$ -subunit CTP linker comprising O-linked glycosylation sites, had more potent activity than the wild-type *in vitro*. Taken together, this new eel LH analog can be produced in large quantities using a stable CHO DG44 cell system.

**Keywords:** Eel LH-M; Stable expression; CHO DG44 cells; cAMP response; pERK1/2

## 1. Introduction

Luteinizing hormone (LH), a member of the glycoprotein hormone family, is composed of a common  $\alpha$ -subunit and unique  $\beta$ -subunits [1,2]. Chorionic gonadotropin (CG)  $\beta$ -subunit is specifically secreted from the placenta of primates and horses during early pregnancy and evolved from the LH  $\beta$ -subunit gene [3]. Human CG (hCG)  $\beta$ -subunit and hLH  $\beta$ -subunit genes are found to differ; however, a single gene encodes both the CG  $\beta$ -subunit and LH  $\beta$ -subunit in horse [4–9]. These glycoproteins are secreted from the pituitary gland and control gonadal function in mammals and fish species [10,11]. Eel LH  $\alpha$ - and  $\beta$ -molecules are bound together via noncovalently linked subunits,

which comprise 93 and 116 amino acids, respectively. The eel  $\alpha$ -subunit has two N-linked glycosylation sites at positions Asn<sup>56</sup> and Asn<sup>79</sup>; these glycosylation sites are highly conserved. The LH  $\beta$ -subunit has one glycosylation site at Asn<sup>10</sup>, as found in most mammals [12].

The oligosaccharide chains in LH, follicle-stimulating hormone (FSH), equine CG (eCG), and hCG are very important for cAMP/PKA signal transduction via receptors [13–17]. In fact, the glycosylation sites in eCG, eel FSH, and eel LH are suggested to be indispensable for biological activity through receptors in vitro and in vivo [11,18,19]. Rec-eel LH and FSH proteins are produced in the silkworm system; however, the *Bombyx mori* system does not have excellent biological activity for glycosylation, a post-translational modification, despite large quantities of rec-proteins [20,21]. Almost the same results are obtained in *Drosophila* S2 cells [22] and *Pichia pastoris* [23,24]. These glycoproteins can be produced in Chinese hamster ovary (CHO)-K1 and CHO-suspension (CHO-S) cells; however, they cannot be produced on large scale [2,18,19,25,26].

Owing to glycoprotein hormones, such as hCG, FSH, LH, and TSH, the CG  $\beta$ -subunit carboxy-terminal peptide (CTP) linker was found to induce substantially increased in vivo potency and circulatory half-lives [27–32]. Recently, eCTP linker attachments in tethered eel LH-wt and FSH-wt caused early secretion and signal transduction [25]. Remarkable results have been obtained in clinical trials with the hGH-MOD-4023 molecule, fused to hGH via the hCG  $\beta$ -subunit CTP linker (117-145 aa) [33–36].

Luteotropin/chorionic gonadotropin hormone receptor (LH/CGR) is a member of the superfamily of G protein-coupled receptors (GPCRs) and is involved in receptor-mediated responses, such as the cAMP response [37,38] and pERK1/2 activation [39–41]. Gas proteins and  $\beta$ -arrestins are related to ERK signaling via two temporally distinct mechanisms: G protein-dependent mechanism, which is rapid in onset, and  $\beta$ -arrestin-dependent mechanism, which is slower in onset [42–44].  $\beta$ -arrestins are involved in pERK1/2 with  $\beta$ -arrestin 1 and 2 in FSHR [45,46]. In terms of eel FSHR, pERK1/2 activation was found to be very fast, occurring 5 min after agonist stimulation [47,48]. Thus, eel LH should also be examined to elucidate its involvement in pERK1/2 activation and the cAMP signal response.

CHO DG44 cells are reported to be a good model for producing large quantities of rec-therapeutic proteins, such as erythropoietin [49],  $\alpha$ -thrombin [50],  $\alpha$ -1 antitrypsin [51], hFSH [52], hCG [53], and eCG [54,55]. Japanese eel is one of the most important fish species in East Asian countries. In general, the most common method for inducing maturation in female eels involves weekly injection of freeze-dried pituitaries from Pacific salmon (SPE) and carp (CPE) [12,20,22]. Notably, SPE treatment does not completely induce oocyte maturation and ovulation and is considered a complete artificial aquaculture system for hormone composition. Thus, CHO DG44 cells were selected to produce large quantities of eel LH-M. As a result, attaching an eCTP linker between the  $\beta$ -subunit and  $\alpha$ -subunit was found to induce more potent biological activity and longer half-life.

Overall, a new potent hormone was found to be essential for artificial maturation, with rec-eel LH-M exhibiting a more potent biological activity in vitro. Based on our results, a new rec-eel LH-M protein was continuously produced using a mass system in CHO DG44 cells.

## 2. Materials and Methods

### 2.1. Materials

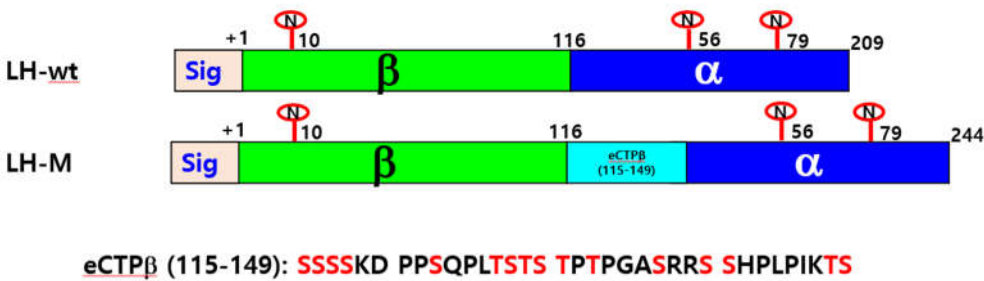
The oligonucleotides were synthesized by Genotech (Daejeon, Korea). The pGEM-T easy cloning vector was purchased from Promega (Madison, WI, USA). The pOptiVEC™-TOPO TA Cloning kit, CHO DG44 cells, CD DG44 medium, CD FortiCHO™ medium, CD OptiCHO™ medium, Freedom™ DG44 Kit, methotrexate (MTX) reagent, and cloning media were purchased from Life Technologies (Carlsbad, CA, USA). Freestyle MAX reagent and Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA, USA). CHO-K1 cells and HEK 293 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The mammalian expression vector, pCORON1000 SP VSV-G, was purchased from Amersham Biosciences (Piscataway, NJ, USA). CHO-S-SFM II medium, fetal bovine serum (FBS), Ham's F-12 medium, and OptiMEM were purchased from Gibco (Grand Island, NY, USA). The monoclonal antibodies for western blotting and ELISA were produced in our

laboratory [12] and labeled with horseradish peroxidase (HRP) by Medexx, Inc. (Seongnam, Korea). The PNGase F kit was purchased from New England Biolabs (Ipswich, MA, USA). The cAMP-homogeneous time-resolved fluorescence (HTRF) assay kit was purchased from Cisbio (Codolet, France). The Lumi-Light<sup>plus</sup> western blotting substrate was obtained from Roche Inc. (Pleasanton, CA, USA). The pERK1/2 antibody, total ERK1/2 antibody, and goat anti-mouse HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). SuperSignal<sup>TM</sup> West Pico PLUS Chemiluminescent substrate was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). DNA ligation reagents, endonucleases, polymerase chain reaction (PCR) reagents, and restriction enzymes were purchased from Takara Bio (Shiga, Japan). The QIAprep-Spin plasmid kit was purchased from Qiagen Inc. (Hilden, Germany), and disposable spinner flasks were purchased from Corning Inc. (Corning, NY, USA). Centriplus Centrifugal Filter Devices were purchased from Amicon Bio Separations (Billerica, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Vector Construction of Eel LH-wt and LH-M

We constructed single chain eel LH-wt a with linked  $\alpha$ -subunit without the signal sequence at the C-terminal region of the LH  $\beta$ -subunit, as described previously [11]. The LH-M mutant was also constructed by attaching an eCG  $\beta$ -subunit CTP region between the  $\beta$ -subunit and  $\alpha$ -subunit via PCR, as described previously [25]. The full-length PCR products were ligated into the pGEMT-Easy vector. The full-length fragments of eel LH-wt and LH-M mutant were ligated into the pOptiVEC TOPO TA Cloning expression vector. Finally, the direction of insertion was confirmed via restriction enzyme cutting and sequencing for genetic verification. Figure 1 shows a schematic of eel LH-wt and the LH-M mutant model, which has eCTP between the  $\beta$ -subunit and  $\alpha$ -subunit.

Figure 1



**Figure 1.** Schematic of rec-eel LH-wt and eel LH-M. The tethered form of eel LH  $\beta/\alpha$ -wt was engineered to contain the  $\beta$ -subunit and common  $\alpha$ -subunit sequences. The eCG  $\beta$ -subunit carboxyl-terminal peptide linker was inserted between the  $\beta$ -subunit and  $\alpha$ -subunit in the eel LH-M mutant using polymerase chain reaction. The eCG  $\beta$ -subunit CTP linker has 35 amino acids (115–149aa) and approximately 12 O-linked oligosaccharide sites. The numbers indicate the amino acids of the mature protein, except for the signal sequences. The encircled “N” denotes N-linked glycosylation sites at the eel LH  $\beta$ - and LH  $\alpha$ -subunits, respectively. Green, FSH  $\beta$ ; blue,  $\alpha$ -subunit; and skyblue, eCG  $\beta$ -subunit CTP linker. eCTP  $\beta$  (115–149) represents the amino acid sequences of the eCG  $\beta$ -subunit CTP linker. Red in the eCTP  $\beta$  (115–149) denotes potential O-linked oligosaccharide sites.

2.3. Transfection into CHO DG44 Cells

Expression plasmids were linearized via Pvu 1 restriction enzyme cut and then transfected into CHO DG44 cells using FreeStyle<sup>TM</sup> MAX reagent, according to the supplier’s protocol. Briefly, one day prior to transfection, CHO DG44 cells were passaged at a density of  $3 \times 10^5$  cells/mL. On the day



of transfection, cell density was approximately  $5 \times 10^5$  cells/mL. For each transfection,  $1.5 \times 10^7$  of viable CHO DG44 cells were transfected into a new 125 mL spinner flask. Pre-warmed, complete CD DG44 medium was added to the flask to a final volume of 30 mL. The plasmid DNA (18  $\mu$ g) and 15  $\mu$ L of FreeStyle™ MAX Reagent were mixed with OptiPRO™ SFM. The diluted samples were then incubated for 10 min at room temperature to induce complex formation. The DNA-FreeStyle™ MAX reagent complex was added dropwise to the cells, with slow swirling of the flask.

#### 2.4. Single Cell Isolation and Production of the Rec-Eel LH-wt and LH-M Proteins

At 48 h after transfection, the cells were transferred into complete CD OptiCHO™ medium supplemented with 8 mM of L-glutamine. Fresh growth medium was replaced every 3–4 days for approximately 10–14 days. The cells were adapted for amplification by increasing the amount of MTX reagent. MTX-amplified cells were grown in fresh growth medium without 8 mM L-glutamine. On the day of cloning, the diluted cells were dispensed at 0.5–2 cells per well into a 96-well plate. After isolation, single-cell colonies were transferred to 24-well plates, 6-well plates, and T-25 flasks. Finally, the clones were expanded in 125 mL shaker flasks at 37 °C and 8% CO<sub>2</sub>, with shaking at 130–135 rpm. To confirm protein production, single-clone cells were cultured in 30 mL of fresh medium supplemented with 4 mM L-glutamine. The supernatant was collected and analyzed to determine the presence of the rec-LH-wt and LH-M proteins. Finally, the culture media were collected on days 9 post-seeding and centrifuged at  $100,000 \times g$  for 10 min at 4 °C to remove cell debris. The supernatant was collected and frozen at -80 °C.

#### 2.5. Quantitation and Western Blotting of the rec-Eel LH Proteins

The collected proteins were quantified via double-sandwich enzyme-linked immunosorbent assay (ELISA) in plates coated with a monoclonal antibody, as described previously [11]. One hundred microliters of the medium were added to the plates, which were then incubated for 1–2 h at room temperature. Thereafter, HRP-conjugated anti-eel antibody was added for 1 h. The wells were washed 5 times and 100  $\mu$ L of substrate solution (tetramethylbenzidine) was added to the wells for 20 min. The absorbance of each well was measured at 450 nm using a microplate reader (Cytation 3; BioTek, Winooski, VT, USA).

For western blot analysis, the collected sample (20  $\mu$ L) was subjected to 12% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a membrane, blocked with 5% skim milk, and then incubated with monoclonal antibody for overnight. After washing with TBS-T, the membrane was incubated with a HRP-conjugated anti-mouse secondary antibody for 2 h, followed by 2 mL of Lumi-Light substrate solution for 5 min. Detection was performed using an enhanced chemiluminescence system.

To enzymatically release N-linked oligosaccharides, modified glycans were removed from rec-proteins via treatment with the N-glycosylation enzyme. Briefly, the rec-protein (15  $\mu$ g) was incubated for 1 h at 37 °C with PNGase F [1  $\mu$ L enzymes (2.5 U/mL)/20  $\mu$ L sample + 2  $\mu$ L of 10  $\times$  Glycobuffer + 2  $\mu$ L of 10% NP-40] after boiling at 100 °C for 10 min with 1  $\mu$ L of 10  $\times$  Glycoprotein Denaturing Buffer. The samples were analyzed using SDS-PAGE followed by western blot analysis.

#### 2.6. Analysis of cAMP Levels via Homogenous Time-Resolved Fluorescence Assays

cAMP accumulation in CHO-K1 cells expressing eel LH/CGR was measured using a cAMP Dynamics 2 competitive immunoassay kit. Briefly, rec-LH ligand (5  $\mu$ L) was added to each well and incubated for 30 min. The cryptate-conjugated anti-cAMP monoclonal antibody and d2-labeled cAMP reagent were added to each well, then incubated for 1 h at room temperature. The compatible HTRF energy transfer (665 nm/620 nm) was measured using a TriStar2 S LB942 microplate reader (BERTHOLD Tech, Wildbad, Germany). The cAMP concentration for Delta F% values was calculated using the GraphPad Prism software (version 6.0; GraphPad Software Inc., La Jolla, CA, USA).

#### 2.7. Phospho-ERK1/2 Time Course

The pCORON1000 SP VSV-G plasmid containing eel LHR was transfected into HEK293 cells. After 48 h, the cells were starved for at least 4–6 h and then treated with an agonist. Cells were lysed using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of cellular extracts were loaded onto 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. pERK1/2 and total ERK1/2 were detected via immunoblotting using rabbit polyclonal anti-phospho-p44/42 MAPK (1:2,000) and anti-MAPK1/2 (1:3,000), respectively. The membranes were then incubated with an anti-rabbit secondary antibody. Chemiluminescence was detected using SuperSignal™ West Pico reagent, and phosphorylated ERK1/2 immunoblots were quantified via densitometry using Image-Lab (Bio-Rad, Hercules, CA, USA).

## 2.8. Data Analysis

Dose-response curves were generated for experiments performed in duplicate. GraphPad Prism 6.0 (San Diego, CA, USA) was used to analyze the cAMP response, EC<sub>50</sub> values, and stimulation curves. Curves fitted in a single experiment were normalized to background signals measured in mock-transfected cells. The pERK1/2 values were calculated using GraFit Version 5 (Erithacus Software, Horley Surrey, UK). The results are expressed as mean ± standard error of the mean of three independent experiments.

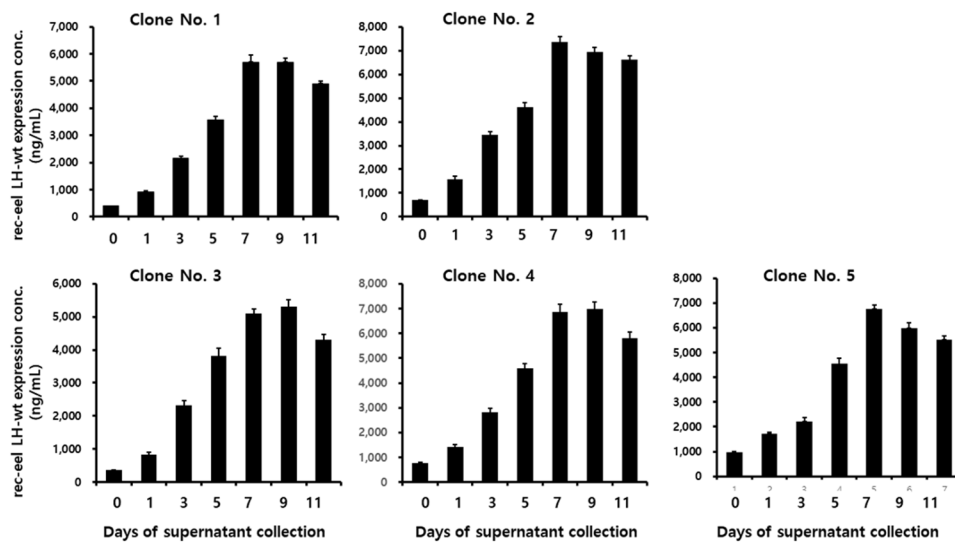
## 3. Results

### 3.1. Isolation of Single Cells after Transfection

Transfected cells were selected at the first stage using CD-OptiCHO medium for approximately 2–3 weeks. The viability of cell transfected with eel LH-wt and eel LH-M decreased gradually to 36% and then continuously increased to 58%, 78%, and >90%. The selected cells were subjected to the second round of selection using the MTX reagent. Cell viability decreased to 40–50% due to the first treatment with 500 nM MTX. The lowest viability (16–31%) was obtained in 2 weeks via treatment with 500 nM MTX. Thereafter, a modest increase was recorded for another two weeks. The MTX concentration was increased by 2 µM to boost the gene copy number. However, cell viability did not decrease below 80%, indicating a slight decreased with recovery to over 90% in two weeks (data not shown). The MTX-selected cells were then isolated using complete cloning medium in 96-well plates. Finally, monoclonal cells expressing rec-eel LH-wt and eel LH-M were isolated from five and nine clones, respectively.

### 3.2. Secreted Quantity of the rec-eel LH-wt Protein

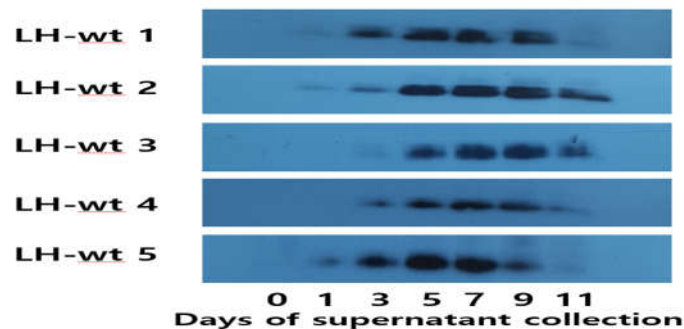
The quantity of the rec-eel LH-wt protein secreted from five clones were determined using ELISA. Supernatants were collected on days 1, 3, 5, 7, 9, and 11 post-cultivation. Rec-eel LH-wt was low on day 1 post-culture and started to increase on day 3. The concentrations gradually increased until the final supernatant was collected, as shown in Figure 2. The amount of rec-LH-wt ranged from 2,150 to 3,450 ng/mL on day 3. The expression levels increased to 3,578–4,625 ng/mL on day 5. High amounts of  $7,346 \pm 246$  and  $6,868 \pm 298$  ng/mL were detected in clones 2 and 4 on day 7, respectively. The secreted quantities of both clones were highest on days 7 and 9; however, high concentrations were consistently observed until day 11 for all clones. These results indicated that the expression level of eel LH-wt gradually increased with cultivation time, with optimal recovery on day 9 in CHO-DG44 cells.

**Figure 2**

**Figure 2.** Quantity of rec-eel LH-wt secreted from CHO DG44 cells based on the day of culture. Supernatant was collected on days 0, 1, 3, 5, 9, and 11 of culture. The expression levels of the rec-eel LH-wt protein was analyzed using a sandwich enzyme-linked immunosorbent assay, as described in the Methods section. Data for five clones are presented as representatives. Values are expressed as mean  $\pm$  standard error of mean of at least three independent experiments.

### 3.3. Western Blotting for rec-eel LH-wt

The molecular weight of rec-eel LH-wt was determined via western blot analysis using the anti-eel glycoprotein hormone  $\alpha$ -subunit monoclonal antibody, as described previously [12]. The collected supernatants (20  $\mu$ L) were analyzed using western blotting. Specific bands were detected at 32–36 kDa in all samples (Figure 3) and weak bands were detected in all clones on day three. The band intensity steadily thickened, and the strongest signal was observed on days 7–9, with a slight decrease on day 11. According to the ELISA and western blot results, optimal recovery occurred on days 7 and 9.

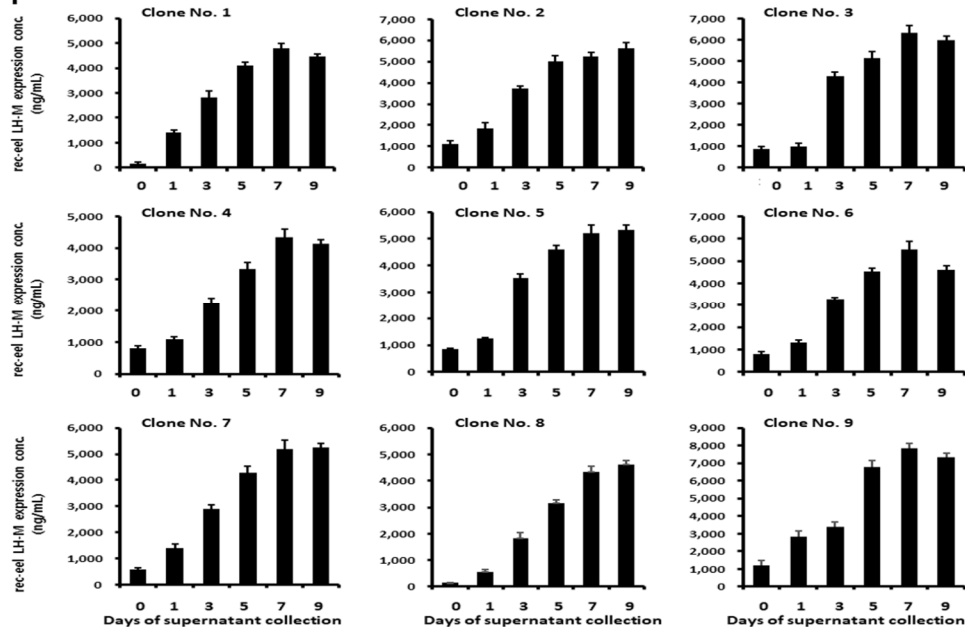
**Figure 3**

**Figure 3.** Western blot analysis of the rec-eel LH-wt proteins produced from single cells. Supernatants from the 5 colonies were collected on the day of cultivation. Five colonies (eel LH-wt 1 to LH-wt 5) were selected and 20  $\mu$ L of supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then blotted onto a membrane and detected using a monoclonal antibody (anti-eel  $\alpha$ -subunit) and HRP-conjugated goat anti-mouse IgG antibodies.

### 3.4. Secreted Quantity of the rec-eel LH-M Protein

To analyze the quantity of secreted proteins in the nine clones of the eel LH-M mutant, the supernatant was collected on days 1, 3, 5, 7, and 9 post-cultivation. The quantity of the rec-eel LH-M protein gradually increased as shown in Figure 4. Based on the clones, 543 to 2,847 ng/mL of the secreted protein was detected on day 1. The secreted pattern was almost the same as that detected in eel LH-wt. The secreted levels rapidly increased to 3,332–6,763 ng/mL on day 5 and reached 4,341 to 7,865 ng/mL on days 7 and 9. The expression was maintained until day 9. The highest expression level of  $7,865 \pm 278$  ng/mL was obtained on days 7 in clone 9. The secreted quantities were high on days 7 and 9 post-cultivation in all clones.

**Figure 4**

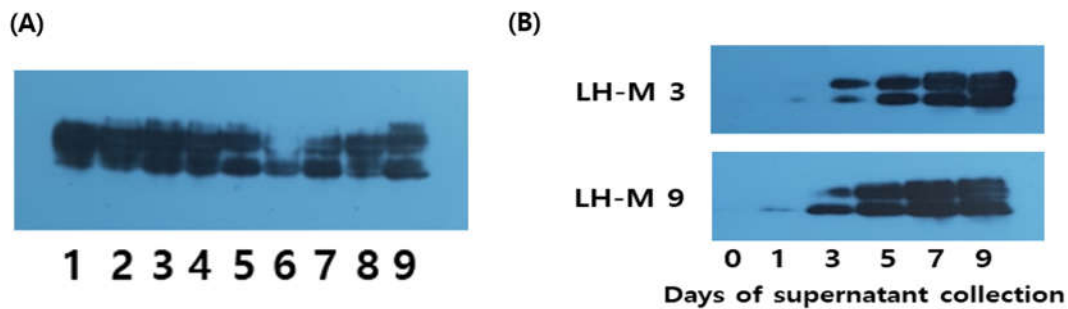


**Figure 4.** Quantity of the rec-eel LH-M proteins secreted from CHO-DG44 cells on the day of culture. Supernatants were collected on days 0, 1, 3, 5, 7, and 9 post-cultivation. Nine clones were isolated, and their expression levels were determined in a time-dependent manner. The expression level was analyzed using a sandwich enzyme-linked immunosorbent assay, as described in the Methods section. Values are expressed as mean  $\pm$  standard error of mean of at least three independent experiments.

### 3.5. Western Blotting for eel FSH-M

The eel LH-M protein was isolated nine clones. Briefly, 20  $\mu$ L of supernatant was collected on day 9 for western blotting. Two specific bands were detected at 38–44 kDa as shown in Figure 5A. The molecular weight of rec-eel LH-M increased by approximately 6–8 kDa compared to that of eel LH-wt. The addition of the eCTP  $\beta$ -subunit linker plays a pivotal role in glycosylation. Thus, the secretion pattern was detected according to the culture time of clones 3 and 9, which indicated gradual increases in the band intensity (Figure 5B). Weak bands were detected on day 3, and strong signals were detected on days 7 and 9, indicating the strong detection of two specific bands.

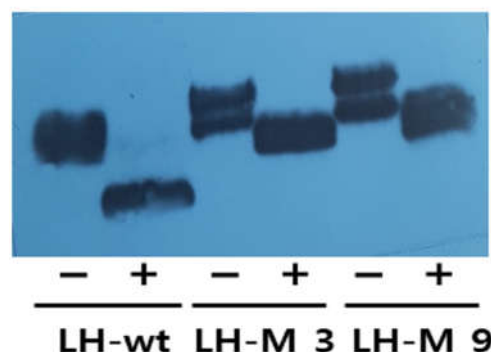


**Figure 5**

**Figure 5.** Western blot analysis of the rec-eel LH-M proteins produced from monoclonal cells. Supernatants from nine colonies were collected on the day of cultivation. The samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and membranes were detected using specific monoclonal antibodies (anti-eel  $\alpha$ -subunit). (A) 20  $\mu$ L of sample collected on day 9 was loaded into the wells. Two specific bands were detected in all samples. (B) Two colonies (eel LH-M 3 and LH-M 9) were selected for western blot analysis and 20  $\mu$ L of the supernatant collected on the day of culture was used for western blot analysis.

### 3.6. Deglycosylation of rec-eel LH-wt and LH-M Proteins

The molecular weights of rec-eel LH-wt and the LH-M mutant were approximately 32–35 and 38–44 kDa, respectively, as shown in Figure 6. However, treatment of the rec-eel LH-wt with PNGase F reduced its molecular weight to approximately 24–28 kDa, indicating a decrease of approximately 8–9 kDa. To further characterize rec-eel LH-M, treatment with PNGase F enzyme markedly decreased the molecular weight to 32–36 kDa. The eCTP  $\beta$ -subunit linker region only has 35 amino acids, including 12 potential O-linked glycosylation sites. Therefore the 6–8 kDa increase in molecular weight indicates partially modification of additional oligosaccharides in the O-linked glycosylation sites. Thus, the carbohydrate content of rec-eel LH-wt and eel LH-M proteins from CHO DG44 cells was appropriately modified.

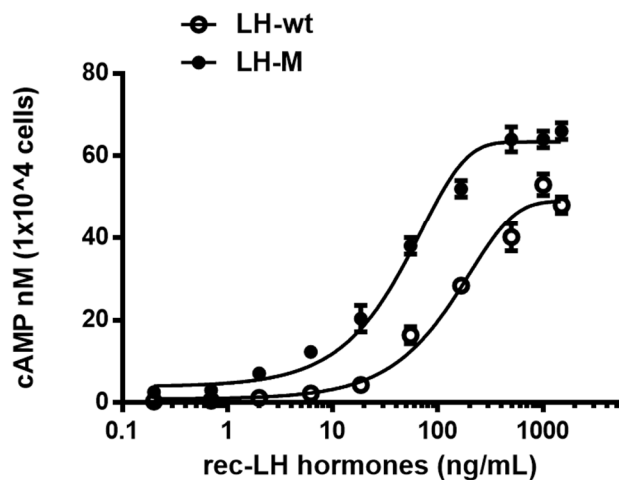
**Figure 6**

**Figure 6.** Deglycosylation of the eel LH-wt and LH-M proteins. The proteins from eel LH-wt, LH-M 3, and LH-M 9 were treated with peptide-N-glycanase F to remove N-linked oligosaccharides and western blot analysis was performed. The molecular weights of the rec-eel LH-wt and LH-M proteins decreased by approximately 8–10 kDa.

3.7. Biological Activities of the rec-eel LH-wt and LH-M Proteins

The in vitro biological activity was assessed using transfected CHO-K1 cells expressing the LH receptor. The potency of cAMP activation in rec-eel LH-wt and LH-M mutant is shown in Figure 7. The dose-response curve of the eel LH-M mutant slightly shifted to the left compared to that of eel LH-wt. Rec-eel LH-wt exhibited full biological activity, with an EC<sub>50</sub> value of 138.8 ng/mL and R<sub>max</sub> of 49.0 ± 1.2 nM/10<sup>4</sup> cells. However, the biological activity of eel LH-M was higher than that of LH-wt, with an EC<sub>50</sub> and R<sub>max</sub> values of 47.8 ng/mL and 63.4 ± 1.5 nM/10<sup>4</sup> cells (Table 1), indicating 2.9- and 1.29-fold increase, respectively. This increase enables the attachment of eel LH-M to the eCG β-subunit CTP linker containing approximately 12 O-linked glycosylation sites.

Figure 7



**Figure 7.** Effect of the rec-eel LH-wt and LH-M proteins on cyclic adenine monophosphate (cAMP) production in cells expressing the eel luteinizing hormone receptor. CHO-K1 cells transiently transfected with eel LHR were seeded in 384-well plates (10,000 cells/well) at 24 h post-transfection. The cells were activated with the rec-eel LH-wt and LH-M proteins for 30 min at room temperature. cAMP production was detected using a homogeneous time-resolved fluorescence assay and presented as Delta F%. cAMP concentrations were calculated using GraphPad Prism software. Each data point represents mean ± standard error of mean of triplicate experiments. The mean data were fitted to an equation to derive a single-phase exponential decay curve.

**Table 1.** Bioactivity of rec-eel LH proteins in cells expressing eel LH receptor.

rec-LH hormones	cAMP responses		
	Basal <sup>a</sup> (nM / 10 <sup>4</sup> cells)	Log (EC <sub>50</sub> ) (ng/mL)	Rmax <sup>b</sup> (nM / 10 <sup>4</sup> cells)
LH-wt	0.9 ± 0.5	138.8 (116.6 to 171.4) <sup>c</sup>	49.0 ± 1.2
LH-M	2.1 ± 0.8	47.8 (40.1 to 57.4)	63.4 ± 1.5

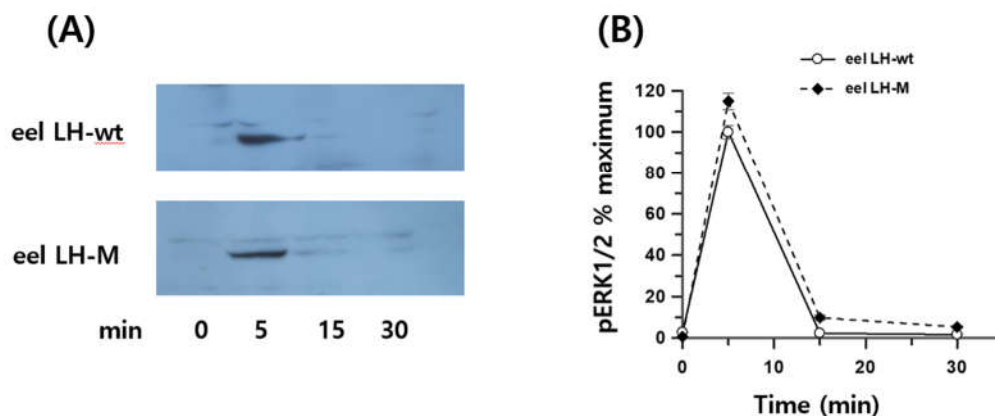
Values are the means ± SEM of triplicate experiments. Log (EC<sub>50</sub>) values were determined from the concentration-response curves from in vitro bioassays. <sup>a</sup> Basal cAMP level average without agonist treatment. <sup>b</sup> Rmax average cAMP level/10<sup>4</sup> cells. <sup>c</sup> Geometric mean (95% confidence intervals) of at least three experiments.

3.8. Phospho-ERK1/2 Activation

MAPK activation sharply increased (within 5 min) in HEK 293 cells transiently transfected to express eel LHR and stimulated with rec-eel LH and rec-eel LH-M (Figure 8). Rapid activation of

pERK1/2 was observed at 5 min, which then decreased abruptly. After agonist stimulation, pERK1/2 activity was highest at 5 min and decreased to approximately 10% of the maximal level at 15 min and 30 min. No difference was observed between the eel LH-wt and LH-M groups. These results indicate that eel LH/CGR by a rec-LH agonist induces PKA-dependent pERK1/2 activation. However, the roles of PKA and PKC in pERK1/2 need to be elucidated.

**Figure 8**



**Figure 8.** pERK1/2 stimulated by the eel FSH receptor. The eel LH receptor was transiently transfected into HEK293 cells, which were starved for 4–6 h and then stimulated with 400 ng/mL agonist for the indicated times. Whole cell lysates were analyzed to determine the levels of pERK1/2 and total ERK. Twenty micrograms of protein were loaded into each sample lane. (A) Western blot results. (B) Western blot results normalized as maximal response % (100% for eel LH-wt at 5 min). The pERK1/2 band was quantified via densitometry, and pERK1/2 was normalized to the level of total ERK. Representative data are shown, and the graphs contain the mean  $\pm$  SE values of three independent experiments. No significant differences were observed between the curves of the eel LH-wt- and LH-M-treated samples.

#### 4. Discussion

Based on the results of the present study, the attachment of an eCG  $\beta$ -subunit-CTP linker between  $\beta$ -subunit and  $\alpha$ -subunit of tethered eel LH significantly increases the biological activity of cAMP responsiveness *in vitro*. Moreover, this new eel LH analog could be produced in large quantities using a stable CHO DG44 cell system. In cells expressing the eel LH receptor, eel LH-M displayed more potent activity in the PKA signal transduction pathway than of eel LH-wt. The eCG  $\beta$ -subunit CTP regions are essential in glycoprotein hormones to achieve early expression, a novel long-acting rec-protein hormone analog, and more potent biological activity.

In the present study, the highest secreted quantity of 5,000–7,500 ng/mL was obtained on day nine post-cultivation. The expression level increased by approximately 10–14-fold compared to the transient expression in CHO-K1 and CHO-S cells, as described previously [11,25]. However, these levels are markedly lower than those obtained in previous studies on human  $\alpha$ -thrombin (1.5 g/L), human  $\alpha$ -1 antitrypsin (1.05 g/L) by methotrexate (MTX) amplification [49–51]. Therefore, the rec-protein quantity in CHO DG44 cells may depend on the gene of interest. A strong band was also detected via western blotting, despite loading of only 20  $\mu$ l supernatant.

Based on western blot analysis, the molecular weight of rec-eel LH-wt and LH-M were 32–36 kDa and 38–44 kDa, respectively. These results are consistent with those of a recent study, in which the same broad band was observed for eel LH-M transiently produced from CHO-S cells, indicating the detection of two specific bands [25]. Therefore, the dimeric eel LH  $\alpha/\beta$  linked with a non-covalent bond from the eel pituitary was approximately 32–38 kDa [56]. Based on these results, broad bands

are widely detected for eel LH-M; therefore, the increased molecular weight was due to attachment of the eCG  $\beta$ -subunit CTP linker. The molecular weight following PNGase F treatment clearly decreased by approximately 8 kDa. These results are almost consistent with those of previous studies, suggesting that the purified eel  $\alpha$ -subunit from eel pituitary reacted with the 17 and 19 kDa proteins; however, the molecular weight decreased to 13 kDa owing to N-glycopeptidase F treatment [22].

In previous studies on hCTP and eCTP linker attachment in glycoprotein hormones, the molecular weight and biological activity of hFSH  $\beta$ -hCTP- $\alpha$ , hCTP-hGH-hCTP-hCTP, hTSH  $\beta$ -hCTP- $\alpha$ , [27,33,57], and eel FSH-M [25] were found to increase. These results are consistent with our findings as the molecular weight of eel LH-M increased by approximately 6–8 kDa. Therefore, the linker containing O-linked glycosylation sites increases the molecular weight, indicating higher PKA/cAMP responsiveness of rec-eel LH-M than eel LH-wt. However, the EC<sub>50</sub> value and maximal cAMP responsiveness stimulation markedly decreased in the deglycosylated eel LH mutants in a dose-dependent manner *in vitro* [11]. Thus, the oligosaccharide chains of the glycoprotein hormones may contribute to the biological activity and molecular weight. hCTP-hGH-hCTP-hCTP was developed as a long-acting human GH for once-weekly administration to GH-deficient adults and children [33,36,58]. We suggest that the eCTP linker attachment contribute to the biological activity and long-acting functions of proteins *in vivo*.

The eCTP linker region contains 34 amino acids, several threonine/serine residues for potential post-phosphorylation, and approximately 12 O-linked glycosylation sites. The O-linked glycosylation induces a specific conformation. According to previous studies, the O-linked glycosylation sites in eCG and hCG play indispensable roles in the secretion of wild-type hormones into the cell supernatant, biological activity, and half-life [1,16,25,59]. However, these investigators also insisted that the hCG CTP regions are not important for receptor binding and signal transduction *in vitro*. Nevertheless, the CTP regions are dispensable for biological activity and half-life *in vivo* [59]. Deletion of the eCG  $\beta$ -subunit from the 34 amino acids, including the O-linked glycosylation sites, significantly delayed the secretion time in CHO-S cells [19]. Thus, attachment of the eCTP linker to the eel LH-M molecule may prolong the circulating half-life of the rec-protein. Therefore, the eel LH-M mutant with eCG  $\beta$ -subunit CTP linker plays a pivotal role in signal transduction through the receptors.

Phosphorylation of ERK1/2 proceeds via the sequential activation of three kinases, Raf1, MEK1, and ERK1/2 [60,61]. The intracellular signaling pathway is activated by LH and includes the Gs-mediated activation of adenylate cyclase, resulting in cAMP production and PKA activation in a cell-specific and G protein-dependent manner [62,63]. This ERK1/2-mediated regulatory process has been observed for many GPCRs that interact with  $\beta$ -arrestins, such as V2 vasopressin receptor [64], parathyroid hormone receptor [65],  $\beta_2$  adrenergic receptor [43],  $\mu$ -opioid receptor [66], neurotensin receptor 1 [44], glucagon-like peptide-1 receptor [67], and angiotensin II [68]. In the present study, pERK1/2 exhibited a peak response 5 min after agonist stimulation. These results are consistent with FSH- and hCG-stimulated pERK1/2 activation in a dose-dependent manner, peak observed at approximately 6 min [46,62]. Based on our results, pERK1/2 is activated in almost the same manner as most GPCRs. Taken together, stimulation of the eel LH/CGR-mediated signaling pathway leads to pERK1/2 activation. pERK1/2 activation by LH/CGR-mediated signaling must be further studied to determine whether a new pathway involving the phosphorylation of downstream effectors of the MAPK pathway exists.

## 5. Conclusions

Herein, rec-eel LH-M was found to exhibit more potent biological activity than eel LH-wt in cells expressing eel the LH/CG receptor. Single cells expressing rec-eel LH-wt and LH-M proteins were isolated from CHO DG44 cells. These cells secreted approximately 15-fold more protein than CHO-S cells. The molecular weight of rec-eel LH-M ranged from 38–44 kDa and two specific bands were detected. The cAMP responsiveness (EC<sub>50</sub> value) in eel LH-M was increased by 2.90-fold, and the maximal responsiveness was 1.29-fold higher than that of eel LH-wt. Similar activation of pERK1/2 levels was recorded with a sharp peak at 5 min, followed by a rapid decline. These findings markedly

important to the production of new potent analogs in large quantities using a stable CHO DG44 cell system. The eCTP linker containing 12 O-linked glycosylation sites needs to exhibit potent activity in vitro and is required for maximal cAMP responsiveness to enable efficient activation of the pERK1/2 signal transduction pathway.

**Author Contributions:** Methodology, M.B.; formal analysis, S.-G.K.; data curation, M.B. S.-P.H. and K.-S.M.; software, S.-H.P.; investigation, M.B.; writing—original draft preparation, B.M.; writing—review and editing, M.-H.K. and K.-S.M.; project administration, M.B., funding acquisition, M.-K.S. and S.-K.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by a research grant from the National Institute of Fisheries Science (R2024050), Republic of Korea.

**Conflicts of Interest:** Author Myung-Hum Park was employed by the company TNT Research. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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