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Biochemical Characterization of Recombinant Equine Chorionic Gonadotropin (rec-eCG), Using CHO Cells and PathHunter Parental Cells Expressing Equine Luteinizing Hormone/Chorionic Gonadotropin Receptors (eLH/CGR)

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Equine chorionic gonadotropin (eCG) consists of highly glycosylated α- and β-subunits and is a unique member of the gonadotropin family, because it elicits the response characteristics of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in species other than the horse. To directly assess the biological function of rec-eCGβ/α, we constructed mammalian expressing vectors of equine luteinizing hormone/chorionic gonadotropin receptors (eLH/CGR). The activity of rec-eCGβ/α *in vitro* assayed in transient transfected CHO-K1 cells and in stably transfected PathHunter Parental cells with eLH/CGR was investigated. rec-eCGβ/α was efficiently secreted in the CHO-K1 suspension cell media, and the quantity detected was about 200 mIU/ml from 1 to 7 days after transfection. In the western blot analysis, the rec-eCGβ/α protein was broadly identified to be about 40~45 kDa molecular weight. The cAMP stimulation in CHO-K1 cells expressing eLH/CGR was determined to evaluate the activity of rec-eCGβ/α. The cAMP concentration increased in direct proportion to the concentration of the rec-eCGβ/α. The EC_{50} value in the transient transfected CHO-K1 cells was 8.1±6.5 ng. The stable cell lines of eLH/CGR were established in the PathHunter Parental cells expressing β-arrestin. We found that rec-eCGβ/α had full LH activity in the PathHunter Parental cells expressing eLH/CGR. The EC_{50} value in transient and stable cells was 5.0±4.7 ng/ml and 4.5±5.2 ng/ml, respectively. These results suggest that rec-eCGβ/α has a biological activity in a cell expressing eLH/CGR. These stable cells expressed in PathHunter Parental cells could be useful for elucidating the functional mechanisms of deglycosylated rec-eCGβ/α mutants.

Key words : cAMP, CHO cells, eLH/CGR, PathHunter Parental cells, rec-eCGβ/α

Introduction

Equine chorionic gonadotropin (eCG) is a member of the glycoprotein hormone family which includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) [20, 25]. eCG is a unique member of the gonadotropin family since it appears to be a single molecule that possesses both LH- and FSH-like activities in other species than the horse [1, 5, 6, 13, 18].

eCG in mares is secreted by the endometrial cups, which binucleate trophoblast that detach from the chorionic girdle of the conceptus, between 37 and120 during pregnancy [3, 8, 11]. eCG appearance coincides with revival of progesterone secretion by the primary corpus luteum (CL) [8]. The formation of the endometrial cups and secretion of eCG initiated subsequently stimulates progesterone synthesis by the primary CL [7]. The primary CL also synthesizes estrone sulfate [9]. High eCG concentrations in mares impregnated by horses protect the CL of pregnancy against the luteolytic effects of $PGF_2α$. Low eCG concentrations carrying mule fetuses afford them less protection against the luteolytic effect of $PGF_2α$, and thus these results may be a cause of the increased foetal mortality that occurs between days 60 and 90 of pregnancy in these mares [11].

Along with LH receptors (LHR), FSH receptors (FSHR) and LH/CG receptor (LH/CGR) belong to a subfamily of glycoprotein hormone receptors that include G-protein-asso-

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ciated seven transmembrane-domain receptors [2, 11]. According, eCG displays dual LH and FSH activities in non-equid species [29]. eCG treatment in dairy cattle results in fewer atretic follicle, the recruitment of smaller follicles (<5 mm) with an increase growth rate, and sustains the growth of medium (6-8 mm) and large (>9 mm) follicles [10, 23]. eCG administration has been related to an increase in the ovulation rate [15, 23] and especially in early post-partum cows [26] and in cows suffering long anoestrus or are under seasonal heat stress [15]. A dairy cows received single dose of 600 IU eCG intramuscularly (i.m.) between 9 and 15 postpartum to increase reproductive performance could not be recommended under the given circumstance [12]. *In vitro*, eCG stimulated luteal cells progesterone production as well as INSR and GLUT protein expression [28]. In sheep, the administered eCG (20 ug/ml) in oocytes was significantly higher FSHR and GnRHR protein levels compared with those matured in the absence of eCG (without eCG) [30]. Thus eCG enhances maturation and decreases apoptosis of oocytes undergoing IVM, and heightens FSHR, LHR, and GnRHR expression [30].

In the recombinant eCG studies, the 102-104 sequence in eCG β-subunit appears to be of utmost importance for their binding to FSHR [5]. The 104-109 region of the eCG β-subunit is essential for the secretion of a fully folded eCGβ/α and for its FSH activity but not for its LH activity [13]. The heterodimeric rec-eCGβ/α exhibits the same thermal stability as natural pituitary LH and its advantages over the eCGβ/α include higher in vitro bioactivity, and reduced potential risk of immunogenicity [18]. We also reported that rec-eCG produced from CHO cells displays dual LH- and FSH- activities in the rat Leydig cells and rat granulose cells [19, 20] and in the rat LH/CGR and rat FSHR cells [16, 24, 25].

In the present study, we constructed the mammalian expressing vector of eLH/CGR and investigated the biological activity of rec-eCGβ/α using cells expressing eLH/CGR in the CHO cells and PathHunter Parental cell lines expressing β-arrestin.

Materials and Methods

Materials

The oligonucleotides used in this study were synthesized by Genotech (Daejon, Korea). The following reagents and materials were also used: restriction enzymes and a DNA ligation kit (Takara, Tokyo, Japan); CHO cells (Japanese Cancer Research Resources Bank, Tokyo, Japan); Ham's F-12 medium, Opti-MEM I, serum-free CHO-S-SFM II, Geneticine and Lipofectamine 2000 (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (Hyclone Laboratories, Logan, UT, USA); Pro-Prep™ protein-extraction solution (Intron Biotechnology, Seoul, Korea); Lumi-Light Western blot kit (Roche, Basel, Switzerland); pcDNA3 mammalian expressing vector, CHO-K1 suspension cells, FreeStyle MAX reagent, and FreeStyle CHO expression medium, pCMV-ARMS1-PK2 expression vector, anti-*myc* antibody, antibiotics and Assay Complete medium (Invitrogen Corporation, San Diego, CA, USA), PathHunter CHO-K1 β-arrestin Parental cell line (DiscoveRx, San Diego, CA, USA), Disposable spinner flask (Corning Incorporated, NY, USA). PMSG ELISA kit (DRG International Inc., Mountainside, NJ). cAMP Dynamic 2 immunosaasy kit (Cisbio Bioassay, France). All other reagents used were from Sigma-Aldrich Corp (St. Louis, MO, USA).

Mammalian expression vector construction of rec-eCGβ/α

To obtain the mammalian expressing vector, the cDNA encoding the full-length eCG β-subunit was fused with the mature protein part of the α-subunit using the method of overlapping PCR mutagenesis as previously report [20, 24, 25]. The same method was used to add *myc*-tag (10 amino acids;) between the first amino acid and the second amino acid of the mature protein in eCG β-subunit as previously report [24]. These PCR fragments were digested with *Eco*RI and *Sal*I enzymes and then ligated into the *Eco*RI and *Xho*I sites of the eukaryotic expression vector pcDNA3 (designated as pcDNA3-eCGβ/α). The direction was confirmed through restriction mapping. Finally, this vector was sequenced completely to confirm the Kozak site, *myc*-tag and PCR errors.

Production of rec-eCGβ/α in CHO suspension cell

The expression of rec-eCGβ/α as described previously [22] was transfected into CHO-S cells by using the FreeSytle MAX reagent transfection method according to the supplier's instructions. Briefly, CHO-S cells were cultured with FreeStyle CHO expression medium at 1×10^7 cells/ 30 ml for 3 days. One day prior to transfection, the cells were passage at $5\text{-}6\times10^5$ cells/ml with CHO expression medium of 125 ml in disposable spinner flask (125 ml). On the day of transfection, the cell density is about $1.2\text{-}1.5\times10^6$ cells/ml. Next, DNA (160 μg) was mixed gently in 1.2 ml of the OptiPRO serum free medium (SFM), and FreeStyle MAX reagent (160

μl) for transfection was mixed gently in 1.2 ml of the OptiPRO serum free medium. Both of the mixed medium was incubated for 5 minutes at RT. After then, the complex (2.4 ml) added to each cell suspension flask. For rec-protein assay, the culture medium was collected each 2 ml on days 1, 3, 5, 6 and 7. Finally, the culture media were collected on day 7 after transfection and centrifuged at 15,000 rpm at 4℃ for 10 min to remove cell debris. Supernatants were collected and frozen at -80℃. And then the samples were concentrated by frozen-dry method and mixed by PBS. A rec-protein was analyzed by Western blot or ELISA.

Analysis of rec-eCGβ/α protein and Western blot analysis

The rec-hormones were quantified PMSG enzyme-linked immunosorbant assay (ELISA) using anti-PMSG monoclonal antibody and enzyme conjugate with couple to horseradish peroxidase and TMB substrate according to the supplier's protocol [25]. The culture media (100 μl) analyzed the samples collected on days 1 3, 5, 6 and 7. After the frozen-dry, the concentrated sample was also measured by mixed 10 to 20 times. After add 50 μl of stop solution to each well in final step, the plate reader determined the absorbance at 450 nm within 30 minutes.

For Western blot analysis, the concentrated sample (10 μg) was subjected to reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmi [17]. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.2 um) using a Bio-Rad Mini Trans-Blot electrophoresis cell. The membranes were washed with 1x Tris-Buffered Saline and Tween 20 (TBS-T) and incubated with primary antibody (anti-myc antibody) diluted 1:5,000. Next, the membranes were reacted with the secondary antibody (goat anti-mouse IgG-HRP) diluted with 1:3,000. Subsequently, membranes were incubated for 1 min with 2-ml of the Lumi-Light substrate solution and, after the substrate solution was removed, the membranes were placed on the Saran wrap, covered with a second piece of the Saran wrap, and exposed on X-ray films for 1-10 min.

Construction of eLH/CGR expression vector

eLH/CGR cDNA was cloned using cDNA of testis and ovary as previously reported [24]. The PCR fragments were ligated into the pcDNA3 mammalian expressing vector by *Eco*R1 and *Xho*1 enzyme sites (designated as pcDNA3-eLH/CGRwt). For the Parental cells, the digested eLH/CGR cDNA by *Nhe*1 and *Sac*1 enzymes were cloned the same enzyme sites of pCMV-ARMS1-PK2 expression vector (designated as pCMV-ARMS1-PK2-eLH/CGRwt). There is no stop codon in the C-terminal region of *Sac*1 enzyme site. The direction was confirmed through restriction mapping.

Transient transfection of CHO-K1 cell and stable transfection of PathHunter CHO-K1 EA-Parental cells

Transfections of CHO cells were done using the liposome tranfection method as previously described [25]. CHO cells were cultured in growth medium [Ham's F-12 media containing penicillin (50 U/ml), streptomycin (50 μg/ml) and glutamine (2 mM) and 10% fetal bovine serum]. CHO cells grow at 80-90% in 6 well plates and the plasmid DNAs were tranfected using lipofectamine reagent. After combined the diluted DNA with lipofectamine reagent, the mixed tube was incubate about 20 mins. CHO cells were washed by Opti-MEM and DNA-lipofectamine complex was added to each well. After 5 hr, CHO growth medium included 20% FBS added to each well. The transfected culture medium was changed by new CHO growth medium at 24 hr after transfection. And then the cells were adjusted for cAMP analysis at the 48-72 hr after transfection.

PathHunter CHO-K1 EA-Parental cells, which engineered to stably express the enzyme acceptor-tagged b-arrestin fusion protein, were transiently and stably transfection as supplier protocol. PathHunter CHO-K1 EA-Parental cells were cultured in AssayComplete™ CHO-K1 culture medium [AssayComplete CHO-K1 medium with 10% fetal bovine serum and antibiotics mixed (penicillin, streptomycin and glutamine)]. For the stably cell lines, transfected cells were seeded 500 and 1,000 cells in 100 mm culture dish at 24-48 hr after transfection. The cells cultured in AssayComplete medium containing G418 to isolate the cells expressing eLH/CGR for 2-3 weeks. Approximately 20 clones were recovered and cultured in a 24 well plate. The grown cells were transfer to 6 well plate and 25 cm^2 culture flask. Finally, 5 cell clone lines were isolated and stocked according to the method previously reported [20].

cAMP assay via homogenous time-resolved foster resonance energy transfer (HTRF)

Measurement of AMP accumulation in CHO cells and PathHunter CHO-K1 EA-Parental cells was performed using cAMP Dynamics 2 competitive immunoassay kits (Cisbio

Bioassays) as described previously [4]. The cAMPs assay uses a cryptate-conjugated anti-cAMP monoclonal antibody and d2-labeled cAMP. The tranfected cells of eLH/CHRs were divided 10,000 cells per well into 384 well. And cell dilution buffer was added MIX in order to prevent cAMP degradation. The standard samples prepared to cover an average range of 0.17~712 nM (final concentration of cAMP per well). After the cells (10,000) were seeded into 384 well and 5 µl compounds medium buffer were added to each well. The plate was seal and incubated for cell stimulating at RT for 30 min. And then cAMP-d2 (5 µl) and anti cAMP-cryptate (5 µl) were added to each wells. After the plate was seal and incubated at RT for 1 hr. The plate was read on a compatible HTRF reader. Results are calculated from the 665 nm/620 nm ratio and expressed in Delta F % (cAMP inhibition).

Delta F% = (Standard or sample ratio-sample negative) ×100 / ratio negative

The cAMP concentration for Delta F% value were calculated by GraphPad Prism.

Data analysis

Dose-response curves were fitted with a nonlinear regression, variable slope equation using GraFit 5.0 (Erithacus Software Limited, Surrey, UK) and GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA). Curves fitted in a single experiment were normalized to the background signaling measured for mock-transfected cells (0%). Each sum curve was calculated from at least three independent experiments.

Results

Production of rec-eCGβ/α in CHO-S cells and western blot

First, we examined the production quantity of rec-eCGβ/α in CHO-S cells during culture days. The level of rec-eCGβ/α produced was shown in Fig. 1A. The secreted quantity in media was about 200 mIU/ml from 1 to 7 days after transfection. rec-eCGβ/α expression did not detected on day of tranfection. The expression quantity was detected as 201 mIU/ml on day 1 after transfection. The expression was almost the same pattern from 3 to 7 day during cell culture. These patterns were similar to that of the transient expression in attached CHO-K1 cells. Next, we examined expression of rec-eCGβ/α protein in the western blot analysis.

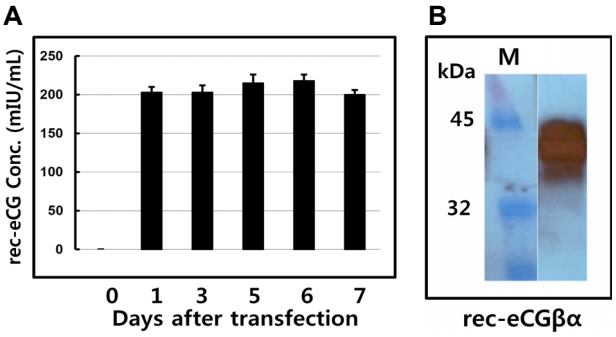

Fig. 1. Quantification of rec-eCGβ/α for transient transfection in CHO suspension cells. A) ELISA results of rec-eCGβ/α. The media were collected and centrifuged on days 1, 3, 5, 6 and 7 after transfection. And then expression quantity of rec-eCGβ/α was analyzed by ELISA as transcribed in Materials and Methods. Values are expressed as mean ± SEM for at least three independent experiments. B) Western blot of rec-eCGβ/α. Sample of rec-eCGβ/α was electrophoresed on 1 12.5% SDS-PAGE. First antibody was used anti-*myc* antibody. The second antibody used goat anti-mouse IgG-HRP. The band of rec-eCGβ/α was detected. M: Marker.

The size of the rec-eCGβ/α protein was identified to be about 40~45 kDa. The band was detected broadly as shown in Fig. 1B. Thus, we suggested that oligosaccharides of about 10~15 kDa were added to the rec-eCG produced in CHO-S cells system.

Biological activity in CHO cells expressing transient eLH/CGR

The effects of the rec-eCGβ/α on cAMP stimulation in CHO cell lines expressing eLH/CGR genes were determined to evaluate the activity of rec-eCG. Receptor cells were incubated with dose-dependent concentrations (0.008~1,500 ng/ml) of rec-eCG. As shown in Fig. 2B, Delta F% in rec-eCG β/α was gradually decreased by the dose-response dependent as standard curve (0.17~712 nM) (Fig. 2A). Here, the cAMP production presented by Delta F% was inhibited by activation of the transfected eLH/CGR (IC_{50} 3.10 ng; Fig. 2B). Next, these data were calculated by cAMP concentration (nM) as shown Fig. 2C. The cAMP concentration increased in direct proportion to the concentration of the rec-eCGβ/α. The EC_{50} value in the transient transfected CHO cells of the eLH/CGR was 8.1±6.5 ng (Table 1).

Biological activity in stably cell expressing eLH/CGR in PathHunter Parental cells

First, eLH/CGR plasmids were transfected into Path-

Table 1. Bioactivity of the rec-eCGb/a between CHO cells and PathHunter Parental cells expressing eLH/CGR

Cells type and transfection method	cAMP responses		
	Basal (nM/10^4 cells)	EC_{50} (ng)	Rmax (nM/10^4 cells)
CHO cells (transient)	1.5±0.2	8.1±6.5	84.4±4.2
Path-Hunter PA CHO cells (transient)	0.6±0.1	5.0±4.7	66.6±5.2
Path-Hunter PA CHO cells (stably)	0.5±0.1	4.5±5.2	74.5±3.9

Values are the means ± SEM of triplicate experiments. The EC_{50} values used to determine the potencies were determined from the concentration-response curves for the *in vitro* bioassays.

Hunter-EA CHO Parental cells expressing β-arresting. Stably clones were selected by G418 treatment. 5 clones were subjected into the cAMP analysis by rec-eCGβ/α (0, 166 and 1,500 ng/ml). As shown in Fig. 3A, the Delta F% value was the most highly inhibited in two cell clones (eLH/CGR_PA_1-3 and 1-5) than other three clones (eLH/CGR_PA_2-9, 2-11 and 2-12). We also calculated the data as cAMP concentration nM as shown in Fig. 3B.

Next, the cAMP stimulation was analyzed between transient cell and stably cell (eLH/CGR_PA_1-3) selected by G418 as shown Fig. 4. Delta F% IC_{50} values in the transient and stably cells (1-3) were 1.0482 ng and 1.0461 ng, respectively. There is no difference between transient and stably cells. The EC_{50} value calculated by cAMP stimulation in both of these cells was 5.0±4.7 and 4.5±5.2 ng, respectively (Table 1). This data suggest for the first time in cells expressing the eLH/CGR. Our data also suggest that rec-eCG displays having a biological activity in cells expressing eLH/CGR. These clone cell could be useful in the biological activity in the deglycosylated rec-eCG mutants.

Discussion

The presented study indicates that rec-eCGβ/α has full activity in the CHO cells and PathHunter Parental cells expressing eLH/CGR. Our results showed that rec-eCGβ/α was efficiently secreted into the medium in the CHO-S cells on day 1 after transfection. The bands of western blot were detected broadly about 40~45 kDa. We also established the stably cells of eLH/CGR in the PathHunter-EA Parental cells expressing β-arrestin which was function as adaptor proteins specifically targeting GPCRs for dynamin-dependent endocytosis via clathrin-coated vesicles. To our knowledge, these results suggest that rec-eCGβ/α has activity in the cells expressing eLH/CGR.

In the secretion pattern of rec-eCGβ/α, we also reported on activity in attached CHO cells [16, 20, 24, 25]. Our results are consistent with those of earlier studies in demonstrating that rec-eCGβ/α in CHO-S cells could be translated and efficiently secreted as biologically active tethered form [20]. Thus, the non-covalent heterodimeric structure is not critical for the glycoprotein hormone family function [24]. On the other hands, the deletion mutant of C-terminal region (Δ87)

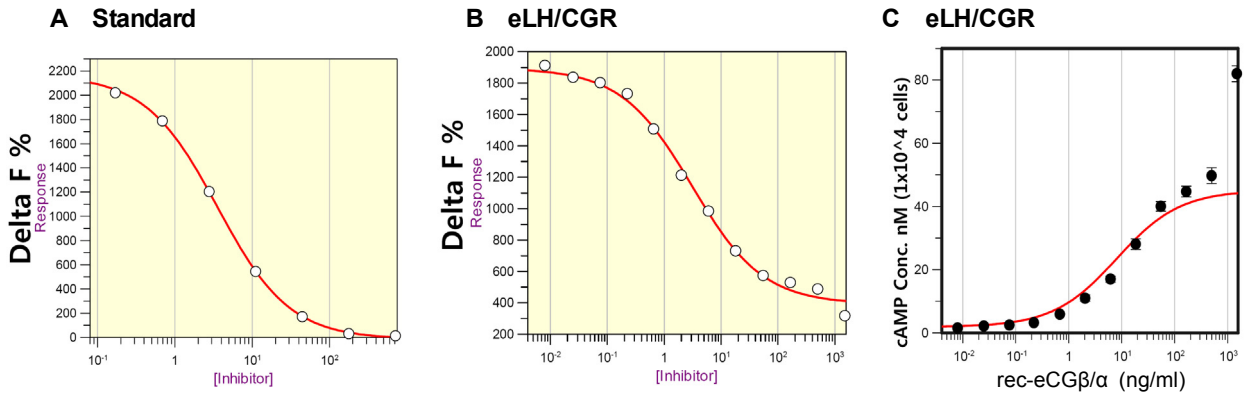

Fig. 2. Dose dependent inhibition and increase in cAMP accumulation induced by a rec-eCGβ/α in transient CHO-K1 cells expressing eLH/CGR. CHO-K1 cells were transfected with eLH/CGR. The transfected culture media were changed by new CHO growth medium at 24 hr after transfection. And then the cells were adjusted for cAMP analysis at the 48-72 hr after transfection (see Materials and Methods for details). A) Standard curve, B) Delta F% value was shown by inhibition (Grafit), C) cAMP nM (1×10^4 cells) value by calculated by GraphPad Prism.

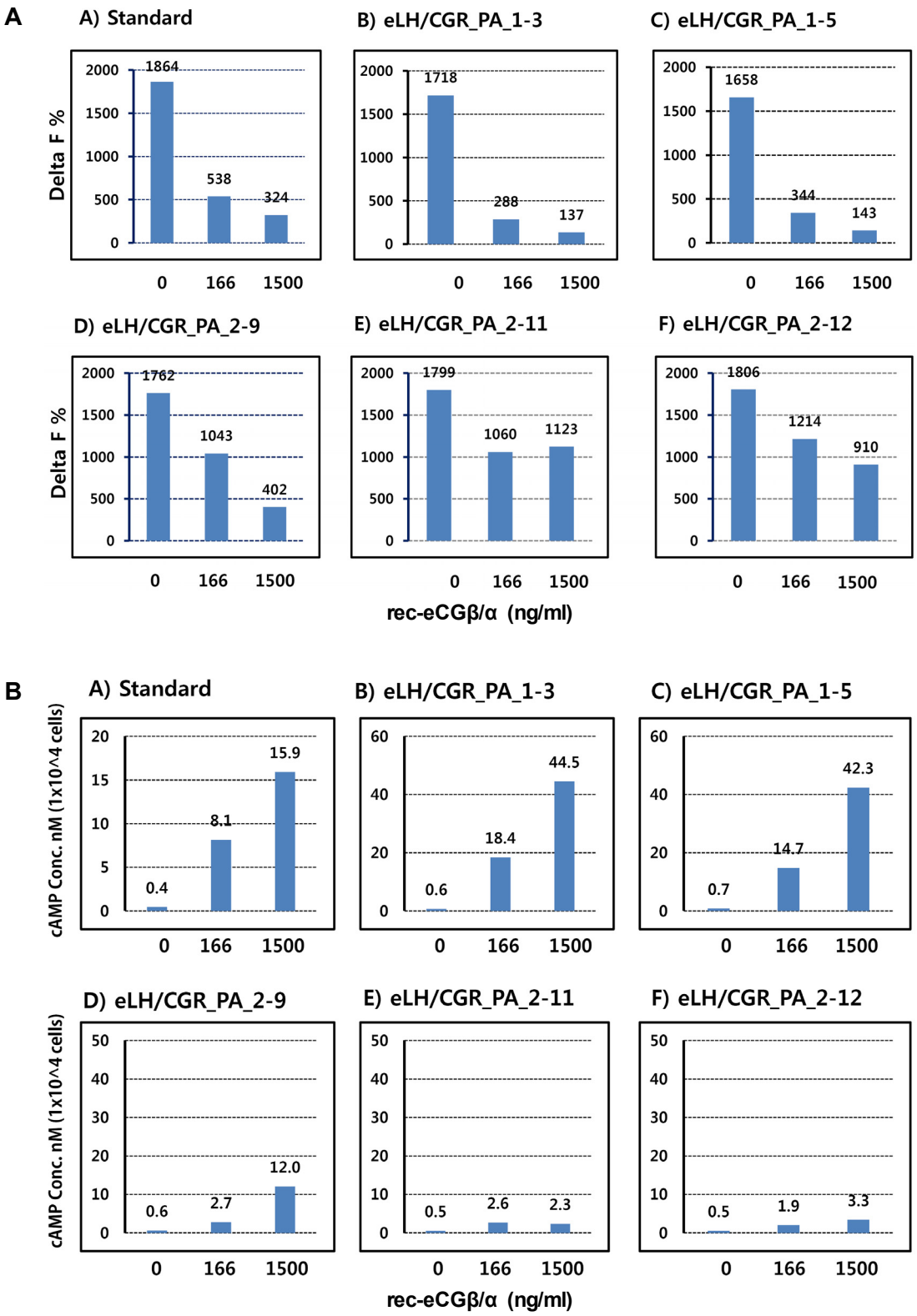

Fig. 3. Selection of stably cells expressing eLH/CGR in PathHunter CHO-K1 EA Parental cells. A: Delta F% value was shown by inhibition. B: cAMP stimulation concentration. PathHunter CHO-K1 Parental cells were cultured in AssayComplete^{TM} CHO-K1 culture medium. After transfection, the cells cultured in AssayCompleteTM medium containing G418 to isolate the cells expressing eLH/CGR for 2-3 weeks. Finally, 5 cell clone lines were isolated and cAMP accumulation analyzed by rec-eCGβ/α concentrations (0, 166, and 1,500 ng/ml). Each point represents the average of three independent experiments.

in the eCG β-subunit did not secreted rec-eCG protein in the medium and. However, mRNA of the Δ87 mutant was transcribed by RT-PCR and northern blot analyses [24]. In the Sf9 system, rec-eCGβ/α was produced 1.5±0.1 ug/ml [18]. The deletion of the 104-149 sequence was the most drastic since no correctly folded hormone was detected in the culture media after 48 hr of expression in the COS-7 cells [13].

In the Western blot results, the band was detected about 40~45 kDa. These results are consistent to the previous studies suggested as approximate size of 43~45 kDa in the attached CHO cells [16, 24, 25]. Our results are consistent that the secreted eCGβ/α single- chain was detected as a doublet of ~46 and 44 kDa in the COS-7 cells [13] and rec eCGβ/α expressed in Sf9 insect cells appear at ~45 kDa and heterodimeric eCG with an upper band at 45 kDa and a lower one at ~38-40 kDa [18]. rec-eCGβ/α produced from milk of transgenic rabbits was also heterogeneous and identified by three bands of 35, 90 and 100 kDa under non-reducing conditions [14]. But the main band of rec-eCGβ/α in the transgenic rabbit was supposed to 35 kDa. Thus, the carbohydrate chains in rec-eCGβ/α were decreased about 10 kDa in the milk of transgenic rabbit. However, rec-eCGβ/α in mammalian cultured cells (CHO-K1, COS7 and CHO-K1 suspension cells) was consistent with the molecular weight observed in these cells.

In the presented study, our results indicate that rec-eCGβ/α displays having a biological activity by a dose-dependent manner in the cells expressing eLH/CGR. In the previous studies, we reported that the heterodimeric rec-eCGα/β showed similar LH- and FSH-like activities to native eCG in the *in vitro* bioassay using primary rat Leydig cells and granulosa cells, respectively [19] and in the tethered-eCGβ/α [20]. We also suggest that rec-eCG has LH- and FSH activities in nonequid species *in vitro* using cells expressing ratLH/CGR and ratFSHR [16, 24, 25]. However, no reports

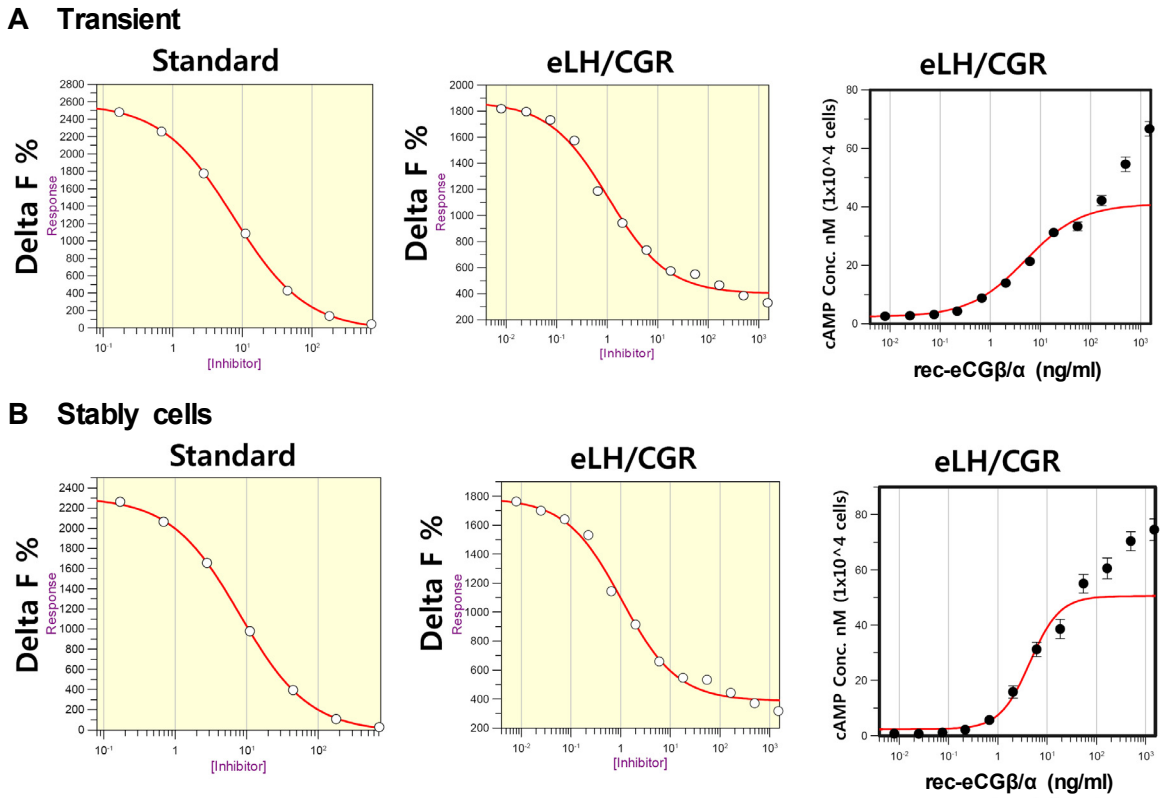

Fig. 4. Dose dependent inhibition and increase in cAMP accumulation induced by a rec-eCGβ/α in transient and stably cells expressing eLH/CGR. PathHunter CHO-K1 EA Parental cells were transfected with eLH/CGR. The transient method described in Fig.2 legend. In the stably cells, cells (eLH/CGR_PA_1-3) were divided 10,000 cells per well into 384 well. The standard samples prepared to cover an average range of 0.17-712 nM. The plate was incubated for 30 min at RT after rec-eCGβ/α adding (0 to 1,500 ng/ml). cAMP d2 and anti cAMP-cryptate were added and incubated at RT for 1 hr. Inhibition of cAMP accumulation was shown by Delta F%. And the data of cAMP concentration was calculated by GraphPad Prism. A) Standard curve, B) Delta F% value was shown by inhibition (Grafit), C) cAMP nM (1×10^4 cells) value by calculated by GraphPad Prism.

have been published about the functions of rec-eCG in the cells expressing eLH/CGR. Thus, the functional significance of rec-eCG protein in the equine must be elucidated. To the best of our knowledge, this is the first study that reported on the biological activity in cells expressing the eLH/CGR. FSH-like activities of two gonadotropins, rec-eFSHβ/α and rec-eCGβ/α, are evoked through the distinct molecular mechanisms regarding the biological role of oligosaccharide at Asn^{56} of the α-subunit [27]. Our results also agree with the finding that the dual activities of rec-eCG displayed in the progesterone stimulation using MLTC-1 cell line from mouse Leydig tumor and using Y1 cell line from a mouse adrenal cortex tumor stably expressing the hFSH receptor [5, 13]. The other groups reported that LH bioactivity of rec-eCG was determined by their steroidogenic activity in MLTC-1 cells expressing an endogenous mouse LH receptor [18].

In conclusion, the eLH/CGR cells expressed in CHO-K1 and PathHunter Parental cells provides us with a valuable model with which to research the function of deglycosylated rec-eCG mutants on the receptor binding and activation in equid. Our results suggest that rec-eCG produced from CHO-K1 suspension cells could be utilize to reproductive physiology in equine. Thus, further investigation of functional significance of rec-eCGs for equids has to be carried out. Further studies are required to elucidate the functional mechanisms that regulate the roles in the ovary and testis of equine.

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References

1. Apparailly, F. and Combarnous, Y. 1994. Role of sialic acid residues in the in vitro superactivity of human choriogonadotropin (hCG) in rat Leydig cells. *Biochim. Biophys. Acta* **1224,** 559-569.
2. Ascoli, M., Fanelli, F. and Segaloff, D. L. 2002.The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr. Rev.* **23,** 141-174.
3. Boeta, M. and Zarco, L. 2012. Luteogenic and luteotropic effects of eCG during pregnancy in the mare. *Anim. Reprod. Sci.* **130,** 57-62.
4. Brule, C., Perzo, N., Joubert, J. E., Sainsily, X., Leduc, R., Castel, H. and Prezeau, L. 2014. Biased signaling regulates the pleiotropic effects of the urotensin II receptor to modulate its cellular behaviors. *FASEB J.* **28,** 5148-5162.
5. Chopineau, M., Martinat, N., Galet, C., Guillou, F. and Combarnous, Y. 2001. β-subunit 102~104 residues are crucial to confer FSH activity to equine LH/CG but are not sufficient to confer FSH activity to human CG. *J. Endocrinol.* **169,** 55-63.
6. Combarnous, Y., Guillo, F. and Martinat, N. 1984. Comparison of *in vitro* follicle-stimulating hormone (FSH) activity of equine gonadotropins (luteinizing hormone, FSH, and chorionic goandotropin) in male and female rats. *Endocrinology* **115,** 1821-1827.
7. Condon, W. A., Ganjam, W. K. and Kenney, R. M. 1979. Catecholamines and equine luteal progesterones. *J. Reprod. Fertil. Suppl.* **27,** 199-203.
8. Conley, A. J. 2016. Review of the reproductive endocrinology of the pregnant and parturient mare. *Theriogenology* **86,** 355-365.
9. Daels, P. F., Shideler, S., Lasley, B. L. and Hughes, J. P. 1990. Source of oestrogen in early pregnancy in the mare. *J. Reprod. Fertil.* **90,** 55-61.
10. Drancourt, M. A., Thatcher, W. W., Terqui, M. and Andrieu, D. 1991. Dynamics of ovarian follicular development in cattle during the estrous cycle, early pregnancy and response to PMSG. *Domest. Anim. Endocrinol.* **8,** 209-221.
11. Flores-Flores, G., Velazquez-Canton, E., Boeta, M. and Zarco, L. 2014. Luteoprotective role of equine chorionic gonadotropin (eCG) during pregnancy in the mare. *Reprod. Dom. Anim.* **49,** 420-426.
12. Freick, M., Passarge, O. and Weber, J. 2017. Lack of effects of an equine chorionic gonadotropin (eCG) administration between days 9 and 15 postpartum on reproductive performance in a Holstein dairy herd. *Reprod. Domest. Anim.* **52,** 429-436.
13. Galet, C., Guillou, F., Foulon-Gauze, F., Combarnous, Y. and Chopineau, M. 2009. The b104-109 sequence is essential for the secretion of correctly folded single-chain ba horse LH/CG and for its activity. *J. Endocrinol.* **203,** 167-174.
14. Galet, C., Menck, Le., Bourhis, C., Chopineau, M., Le Griec, G., Perrin, A., Magallon, T., Attal, J., Viglietta, C., Houdebine, L. M. and Guillou, F. 2000. Expression of a single *βa* chain protein of equine LH/CG in milk of transgenic rabbits and its biological activity. *Mol. Cell. Endocrinol.* **174,** 31-40.
15. Garcia-Ispierto, I., Lopez-Helguera, I., Martino, A. and Lopez-Gatius, F. 2012. Reproductive performance of anoestrous high-producing dairy cows improved by adding equine chorionic gonadotrophin to a progesterone-based oestrous synchronizing protocol. *Reprod. Domest. Anim.* **47,** 752-758.
16. Jeoung, Y. H., Yoon, J. T. and Min, K. S. 2010. Biological functions of the COOH-terminal amino acids of the a-subunit of tethered equine chorionic gonadotropin. *Reprod. Dev. Sci.* **34,** 47-53.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.
18. Legardinier, S., Poirier, J. C., Klett, D., Combarnous, Y. and Cahoreau, C. 2008. Stability and biological activities of heter-

odimeric and single-chain equine LH/chorionic goandotropin variants. *J. Mol. Endocrinol.* **40,** 185-198.
19. Min, K. S., Hattori, N., Aikawa, J. I., Shiota, K. and 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. *Endocr. J.* **43,** 585-593.
20. Min, K. S., Hiyama, T., Seong, H. H., Hattori, N., Tanaka, S. and Shiota, K. 2004. Biological activities of tethered equine chorionic gonadotropin (eCG) and its deglycosylated mutants. *J. Reprod. Dev.* **50,** 297-304.
21. Min, K. S., Liu, X., Fabritz, J., Jaquette, J., Abell, A. N. and Ascoli, M. 1998. Mutations that induce constitutive activation and mutations that impair signal transduction modulate the basal and/or agonist-stimulated internalization of the lutropin/choriogonadotropin receptor. *J. Bio. Chem.* **273,** 34911-34919.
22. Nanjidsuren, T. and Min, K. S. 2014. The transcription factor Ap-1 regulates monkey 20α-hydroxysteroid dehydrogenase promoter activity in CHO cells. *BMC Biotechnol.* **14,** 71.
23. Pacala, N., Corin, N., Bencsik, I., Dronca, D., Cean, A., Boleman, A., Caraba, V. and Papp, S. 2010. Stimulation of the reproductive function at cyclic cows by ovsynch and PRID/ECG. *Anim. Sci. Biotech.* **43**, 317-320.
24. Park, J. J., JarGal, N., Yoon, J. T. and Min, K. S. 2009. Function of the tethered rec-eCG in rat and equine receptors. *Reprod. Dev. Biol.* **33,** 229-236.
25. Park, J. J., JarGal, N., Yoon, J. T. and Min, K. S. 2010. β-subunit 94-96 residues of tethered recombinant equine chorionic gonadogropin are important sites luteinizing hormone and follicle stimulating hormone like activities. *Reprod. Dev. Biol.* **34,** 33-40.
26. Rostami, B., Niasari-Naslaji, A., Vojgani, M., Nikjou, D., Amanlou, H. and Gerami, A. 2011. Effect of eCG on early resumption of ovarian activity in postpartum dairy cows. *Anim. Reprod. Sci.* **128,** 100-106.
27. Saneyoshi, T., Min, K. S., Ma, X., Nambo, Y., Hiyama, T., Tanaka, S. and Shiota, K. 2001. Equine follicle-stimulating hormone: molecular cloning of *β*-subunit and biological role of the asparagine-linked oligosaccharide at asparagines[56] of *α*-subunit. *Biol. Reprod.* **65,** 1686-1690.
28. Sousa, M., Mendes, G. P., Campos, D. B., Baruselli, P. S. and Papa, P. C. 2016. Equine chorionic gonadotropin modulates the expression of genes related to the structure and function of the bovine corpus luteum. *Plos One* **11,** e0164089.
29. Stewart, F. and Allen, W. R. 1981. Biological functions and receptor binding activities of equine chorionic goandotropins. *J. Reprod. Ferti.* **62,** 527-536.
30. Wei, S. C., Gong, Z. D., Zhao, H. W., Liang, H, Q., Lai, L. J. and Deng, Y. Y. 2016. Equine chorionic gonadotropin influence on sheep oocyte *in vitro* maturation, apoptosis, and follicle-stimulating hormone receptor and luteinizing hormone receptor expression. *Genet. Mol. Res.* **15,** doi: 10.4238/gmr15049162.

초록 : 말의 LH/CGR를 발현하는 CHO 세포와 PathHunter Parental 세포에서 유전자 재조합 eCGβ/α의 생화학적 특성

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eCG는 다른 포유동물에서 FSH와 LH의 활성을 나타내기 때문에 성선자극 호르몬 family에서 아주 특이적이고 많은 당쇄가 수식되어진 알파와 베타의 비공유결합으로 구성되어 있다. 유전자 재조합 eCGβ/α의 생물학적 기능을 규명하기 위하여 말의 LH/CGR의 포유동물발현용 벡터를 구축하였다. 재조합 eCGβ/α의 활성분석은 말의 LH/CGR가 일시적으로 발현되는 CHO-K1 세포와 지속적으로 발현되는 PathHunter Parental 세포를 이용하여 분석하였다. 유전자 재조합 eCGβ/α는 CHO-K1 부유세포의 상층으로 효율적으로 분비되었으며, 분비량은 transfection 후 1일에서 7일까지 약 200 mIU/ml이었다. Western blot 분석결과는 재조합 eCGβ/α의 분자량은 약 40-45 kDa으로 검출되었다. eLH/CGR가 발현되는 CHO-K1 세포에서의 cAMP분비량으로 재조합 eCGβ/α의 활성을 분석하였다. 그 결과 cAMP농도는 재조합 eCGβ/α의 농도의존적으로 증가하였다. eLH/CGR가 일시적으로 발현하는 CHO-K1 세포에서 EC_{50} 값은 8.1±6.5 ng이었다. 또한 일시적 및 지속적으로 eLH/CGR가 발현하는 PathHunter Parental 세포에서도 재조합 eCGβ/α의 LH 활성 분석결과 높은 활성을 나타내는 것으로 확인되었으며, 이들의 EC_{50} 값은 각각 5.0±4.7 ng/ml, 4.5±5.2 ng/ml으로 나타났다. 따라서 이러한 결과에 의하면 재조합 eCGβ/α는 말의 LH/CGR가 발현하는 세포에서 생물학적 활성을 나타난다는 것을 확인하였으며, PathHunter Parental 세포에서 지속적으로 발현되는 세포의 확보는 당쇄제거에 의한 재조합 eCG의 돌연변이등에 관한 기능적인 메커니즘을 밝히는데 유용할 것으로 사료된다.