

# Comparative gene expression profiling of mouse ovaries upon stimulation with native equine chorionic gonadotropin (eCG) and tethered recombinant-eCG

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## Research article

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# Abstract

**Background:** Equine chorionic gonadotropin (eCG) induces super-ovulation in laboratory animals. Notwithstanding its extensive usage, limited information is available regarding the differences between the *in vivo* effects of native eCG and recombinant eCG (rec-eCG). This study aimed to investigate the gene expression profiles of mouse ovaries upon stimulation with native eCG and rec-eCG produced from CHO-suspension (CHO-S) cells. eCG and rec-eCG were cloned and transfected into CHO-S cells and quantified. Thereafter, we determined the metabolic clearance rate (MCR) of native eCG and rec-eCG up to 24 h after intravenous administration through the tail vein and identified differentially expressed genes in both ovarian tissues, via quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC).

**Results:** Rec-eCG was markedly up-regulated initially after transfection and maintained until recovery on day 9. Oligosaccharide chains were substantially modified in rec-eCG protein produced from CHO-S cells and eliminated through PNGase F treatment. The MCR was slightly lower for rec-eCG than for eCG, and no significant difference was observed after 60 min. Notwithstanding their low concentration, rec-eCG and native eCG were detected in the blood at 24h post-injection. Microarray analysis of ovarian tissue revealed that 20 of 12,816 genes assessed therein were significantly up-regulated and 43 genes were down-regulated by >2-fold in the group receiving rec-eCG (63 [0.49%] differentially regulated genes in total). The microarray results were concurrent with and hence validated by those of RT-PCR, qRT-PCR, and IHC analyses.

**Conclusions:** The present results indicate that rec-eCG can be adequately produced through a cell-based expression system through post-translational modification of eCG and can induce ovulation *in vivo*. These results provide novel insights into the molecular mechanisms underlying the up- or down-regulation of specific ovarian genes and the production of rec-eCG with enhanced biological activity *in vivo*.

## Background

Equine chorionic gonadotropin CG (eCG) is a unique glycoprotein hormone because it has both luteinizing hormone (LH)- and follicle-stimulating hormone (FSH)-like biological activities [1,2]. Glycoprotein hormones including LH, FSH, and thyroid-stimulating hormone (TSH) consist of non-covalently associated  $\alpha$ - and  $\beta$ -subunits [4-6]. The  $\alpha$ -subunit has an identical primary structure in the same species. However, each  $\beta$ -subunit is species-specific and structurally differs among the species [7,8].

The  $\beta$ -subunits of eCG and equine LH (eLH) have an identical primary structure and was reportedly expressed from the same gene [7,9]. Thus, eCG is a potentially suitable good model to study structure-function relationships among gonadotropins owing to its dual activity [10-12]. Owing to its long half-life in blood, a single dose of eCG, as opposed to multiple doses, is adequate to stimulate ovarian gene expression [13]. Furthermore, eCG and human CG (hCG) together stimulate ovulation in rats and mice

[14,15]. Moreover, eCG administration to cows is reportedly associated with an increase in their ovulation rate [16], particularly in early postpartum calves [17,18].

The glycosylation sites at amino acid residue 52 in the  $\alpha$ -subunit of human FSH (hFSH) [19] and hCG [5] and residue 56 in eCG [7] are important for signal transduction when the cAMP response is impaired, and the binding activity of these hormones increased by 20- to 3-fold [20], concurrent with our previous findings [2,6]. Thus, post-translational glycosylation of glycoprotein hormones plays a pivotal role in receptor-mediated signal transduction. N- and O-linked oligosaccharides at 56 of  $\alpha$ -subunit of eCG and a C-terminal extension (residues 114–149) in the  $\beta$ -subunit were included in vectors expressing eCG to produce rec-eCG and to investigate the role of these regions in the biological activity of eCG.

High-throughput RNA sequencing and microarray analysis are useful during transcriptome profiling and gene expression analysis [21,22]. A microarray contains thousands of millions of complementary DNA fragments or oligonucleotides that hybridize with specific RNA molecules in a sample [22]. A recent study revealed differentially expressed genes (DEGs) upon RNA-seq using ovarian tissue of dairy goats upon repeated eCG treatment [23], indicating that three-time eCG treatment dysregulated several ovarian genes including glucagon, follistatin-related protein 3 (FSTL3), and aquaporin-3 (AQP3), thereby reducing reproductive function.

Thus far, we have attempted to assess the different roles of rec-eCG with respect to their attached oligosaccharides [2,24], glycosylation sites for LH- and FSH-like activity [2], tethered rec-eCGs [6], internalization of rat FSH and LH receptors by rec-eCG [25], and signal transduction through eel FSH and LH receptors by rec-eCG and native eCG [26]. Furthermore, we have analyzed the ovulation rates between native eCG and rec-eCGs in mice [27]. These results suggest that rec-eCGs are induced at markedly lower in nonfunctional oocytes upon deglycosylation. Approximately 20% of non-functional oocytes with native eCG and only 2% with the rec-eCGs have been determined thus far. Numerous studies have reported the effects of a combination of eCG and hCG on reproductive performance and estrous synchronization [13-16]. However, no studies have investigated the effects of native eCG and rec-eCG on gene regulation through RNA-based microarray analysis.

In the present study, we hypothesized that treatment of ovarian tissues with native eCG and rec-eCG results in different DEG profiles. We produced rec-eCG $\beta/\alpha$  proteins in CHO-S cells, characterized their physiological function *in vivo*, and analyzed the difference in gene expression profiles through microarray analysis.

## Results

### Production of rec-eCG and western blot analysis

eCG contains two N-linked glycosylation sites at amino acid positions 56 and 82 in the  $\alpha$ -subunit of eCG. The  $\beta$ -subunit of eCG contains one N-linked glycosylation site at position 13 and approximately 11 O-linked glycosylation sites at the C-terminal region (Fig. 1). Thus, we constructed an expression vector

encoding the tethered eCG mutant, which was linked with the  $\alpha$ -subunit without the signal sequence at the C-terminal region of the  $\beta$ -subunit. Rec-eCG expression levels were markedly increased to  $210 \pm 10.3$  mIU/mL on day 1 after transfection. These levels were consistently maintained until day 9, being  $212 \pm 12.7$ ,  $227 \pm 16.1$ ,  $230 \pm 15.6$ , and  $202 \pm 7.8$  mIU/mL at 3, 5, 7, and 9 days, respectively (Fig. 2a). Rec-eCG was efficiently secreted into the cell culture medium. eCG levels markedly increased initially upon transfection and were maintained until recovery on day 9.

Further, we analyzed the molecular weight of tethered rec-eCG. On western blot analysis, the approximate molecular weight of rec-eCG was 40–46 kDa (Fig. 2b). After deglycosylation with PNGase F, the molecular weights significantly decreased to approximately 30–36 kDa (Fig. 2b). The oligosaccharide chains were substantially modified post-translation in tethered rec-eCG, confirming the loss of the oligosaccharide chains upon PNGase F treatment.

### **Metabolic clearance rates (MCRs) of natural eCG and tethered rec-eCG *in vivo***

To analyze the MCR, eCG was detected in both groups ( $\sim 550$  mIU/mL) in the serum at 1 h after injection, as shown in Fig. 3. Although the MCR was slightly lower in the rec-eCG-treated groups, no significant difference was observed between native-eCG and rec-eCG treatment after 60 min. Their concentration was low ( $\sim 100$  mIU/mL) until 24 h. These results indicate that rec-eCG produced herein had a normal MCR and induced ovulation, as previously described [27].

### **Comparison of ovarian gene expression profiles between groups treated with native eCG and rec-eCG**

Global gene expression profiles were analyzed in mouse ovarian tissue treated with native eCG and rec-eCG via microarray analysis. The ovarian tissues were harvested at 13 h upon combinational treatment (10 IU of eCG followed by 10 IU of hCG after 48 h). Gene expression levels were analyzed via microarray analysis with 12,816 gene probes. Genes showing a  $>2$ -fold difference in expression levels were identified in eight ovaries (native eCG: four, rec-eCG: four). Figure 4 shows the differences in gene expression profiles between the two samples.

Expression profiles of 63 of 12,816 (0.49%) genes differed at least by 2-fold between the native eCG and rec-eCG groups (Fig. 4). Table 1 shows the significantly up-regulated genes ( $>2$ -fold) in rec-eCG-treated ovaries. Of the 20 (0.16%) genes up-regulated in the rec-eCG group, six genes (*Ctsk*, *Crybb1*, *Rbm8a*, *Sectm1b*, *Tex19.2*, and *Rpusd4*) (30%) were up-regulated 3-fold. The number of genes down-regulated by  $>2$ -fold in the rec-eCG-treated ovaries was 43 (0.34%) (Table 2). Seven genes (*Ovgp1*, *BC048546a*, *BC048546b*, *Emb*, *Tmem68*, *Dcpp1*, and *Ltf*) were down-regulated ( $>3$ -fold) among the 43 genes down-regulated in rec-eCG-treated ovaries (Table 2). Only 63 (0.49%) genes were differentially expressed ( $>2$ -fold) in rec-eCG-treated ovaries.

Further, we analyzed the data to gain insight into the biological processes and functions of the DEGs. The distribution of the 63 DEGs (at least 2-fold) between ovaries treated with native eCG and rec-eCG and their distribution in different Gene Ontology (GO) categories were analyzed (Supplementary Material Fig. 1).

GO analysis was performed using the Panther database (<http://www.pantherdb.org>). GO terms “biological process” were the most represented (>4 genes) among rec-eCG-treated ovarian tissue, including “signal transduction (16 genes),” “developmental processes (14),” “protein metabolism and modification (9),” “cell culture and motility (5),” and “nucleoside, nucleotide and nucleic acid metabolism (4).” In category “molecular function,” these genes were classified into 18 subcategories through GO analysis, with the largest number of genes represented in “protease (6 genes),” “signaling molecule (5),” “oxidoreductase (5),” “nucleic acid binding (5),” and “hydrolase (5).” Seven genes were categorized as “molecular function unclassified” (Supplementary Material Fig. 2). The number of classified genes is the number of genes in categories after excluding overlapping categories.

### **Gene expression analysis through quantitative reverse-transcription PCR (qRT-PCR) analysis**

To validate the results of microarray analysis, we performed RT-PCR and qRT-PCR analyses using specific primers (Supplementary Material Table 1) for the 14 genes identified herein (Fig. 5a, b). Among the up-regulated genes identified through microarray analysis of rec-eCG-treated ovaries, six genes, i.e., *Tex19.2*, *Sectm1b*, *Ctsk*, *Gpnmb*, *Sectm1a*, and *Hsd17b1*, were confirmed to be up-regulated through qRT-PCR (Fig. 5a). Among the down-regulated genes, eight genes, i.e., *OVGP1*, *BC048546*, *Tmem68*, *Dcpp1*, *Prkg2*, *Edn2*, *Adamts1*, and *Akr1b7*, were confirmed to be down-regulated by >2-fold in the rec-eCG-treated mouse ovarian tissue, of which seven, i.e., *OVGP1*, *BC048546*, *Tmem68*, *Dcpp1*, *Edn2*, *Adamts1*, and *Akr1b7*, were confirmed to be down-regulated via qRT-PCR analysis (Fig. 5b). Nonetheless, one gene, *Prkg2*, displayed no significant change in expression levels upon qRT-PCR analysis. The fold-change in the expression levels of these genes was consistent with the results of microarray analysis, confirming that the results of qRT-PCR analysis correlated with those of the microarray analysis.

### **Immunohistochemical analysis of ovarian tissue**

To determine the cell types expressing four proteins (HSD17b1, ADAMTS1, Edn2, and OVGP1), immunohistochemical analysis was performed for the same ovarian tissues used for microarray analysis (Fig. 6). Among the up-regulated genes in the rec-eCG-treated ovarian tissue, HSD17b1 was localized in the granulosa cells and theca folliculi. Among the down-regulated genes in rec-eCG-treated ovarian tissue, ADAMTS1, which is required for normal ovulation and is localized in the cumulus oocyte complex during the preovulatory stage, was also localized in granulosa cells. Edn2, which is transiently expressed in granulosa cells immediately prior to ovulatory follicle rupture, was also strongly expressed in the ovarian stroma of a native eCG-treated ovarian tissue. OVGP1, which improves the efficiency of in vitro fertilization and increases the number of fertilized eggs, was weakly expressed in the ovaries after ovulation. These results indicate that the expression of these four proteins was directly correlated with the time of ovulation in mice.

## **Discussion**

This study examined the biological activity of tethered rec-eCG, containing N- and the O-linked oligosaccharide chains and their MCR *in vivo*. Furthermore, this study evaluated differential gene

expression profiles in mouse ovaries stimulated with native eCG and rec-eCG in combination with hCG. The present study shows differences in the up- and down-regulated genes (>2-fold) in ovarian tissues treated with native eCG and rec-eCG.

Thus, far, we have expressed rec-eCG in only CHO-K1 cells and stable CHO-K1 cells under G418 selection [6,25–27]. Hence, levels of secreted rec-eCG at 24 h post-transfection have remained unknown. However, supernatants of the culture media of CHO-S cells were recovered until 9 days after transfection. In the present study, single-chain rec-eCG was markedly up-regulated on day 1 after transfection in CHO-S cells. However, rec-eCG with a C-terminal deletion in the  $\beta$ -subunit was detected at a low concentration on day 1 and 3 post-transfection (data now shown). The present results indicate that the CTP region including approximately 13 O-linked oligosaccharides plays a pivotal role in the early secretion of eCG from cells into the supernatant medium after transfection.

Various studies have reported that rec-eCG proteins lead to the production and secretion of stable heterodimeric eCG in COS-7 cells [28] and infected Sf9 cells [29], with thermal stability similar to that of native pituitary LH [30]. Secreted single-chain eCG in COS-7 cells is detectable as a doublet of 46 and 44 kDa [12]. The present results show that the molecular weight of rec-eCG greatly decreases upon elimination of the N-linked oligosaccharide chains via PNGase F treatment, decreasing the molecular weight to approximately 30–36 kDa. Our results are consistent with those of other studies, suggesting that rec-eCG contained highly modified N-linked glycosylation sites in COS-7 cells and CHO-K1 cells post-translation [12, 27].

Furthermore, rec-eCG mutants deglycosylated through site-directed mutagenesis was markedly low in number (< 2.4%) in nonfunctional oocytes in comparison with native eCG (21.2%) [27]. These results suggest a specific model for ovulation without displaying a long-half-life and to only induce functional oocytes in experimental animals despite using native eCG. Furthermore, the MCR of rec-eCG was somewhat lower than that of native eCG at 10–60 min after injection and was similarly maintained at 2 and 24 h. The present results suggest that the potency of rec-eCG can be assessed at only 10 IU, as previously described [27]. These MCR results suggest that rec-eCG derivatives can be attentively utilized for animal experiments.

Furthermore, we previously reported that rec-eCG exerts dual LH- and FSH-like activity in *in vitro* bioassays involving rat Leydig and granulosa cells, respectively [2, 6]. Moreover, we previously reported that rec-eCG has both LH- and FSH-like activity in cells expressing rLH/CGR and rFSHR [25]. Nevertheless, no studies have examined differential gene expression in ovaries stimulated with native eCG and rec-eCG. We performed gene expression profiling for ovarian tissue through microarray analysis after administration of native eCG or rec-eCG. We identified genes up- and down-regulated by >2-fold. The present results show that 63 genes were up- and down-regulated (0.49% of 12,816 genes in rec-eCG-injected ovaries). These changes in gene expression profiles directly render oocytes nonfunctional upon comparing native eCG-treated and rec-eCG-treated ovaries, suggesting that rec-eCGb/a derivatives used herein can cause slightly aberrant gene expression in the ovaries and produce functional oocytes without

nonfunctional oocytes, in comparison with native eCG-treated ovaries. In the “biological process” category, the largest number of deregulated genes included signal transduction (16 proteins; Supplementary Material Figs. 1 and 2). In contrast, the largest number of genes (6 genes) among the 18 “molecular function” categories were present in “proteases.” Seven genes were categorized as “molecular function unclassified.” We assessed differences in the expression of ovary-specific genes between groups treated with native eCG and rec-eCG through qRT-PCR analysis. The differences in gene expression were confirmed for six genes. These genes, *Tex19.2*, *Sectm1b*, *Ctsk*, *Gpnmb*, *Sectm1a*, and *Hsd17b1*, were specifically over-expressed in rec-eCG-treated ovaries. Among the genes found to be down-regulated on microarray analysis, seven genes were confirmed to be down-regulated through qRT-PCR analysis. These differences should be further assessed through a systematic study.

Immunohistochemical analysis was conducted to determine the cell type responsible for protein expression in the ovaries. We first confirmed that 17 $\beta$ -hydroxysteroid dehydrogenase type 1 (17 $\beta$ -HSD1), which catalyzes the conversion of estrone to estradiol, is primarily localized in ovarian granulosa cells after rec-eCGb/a injection. Our results are consistent with those of another study, showing that 17 $\beta$ -HSD1 is expressed in the placenta and ovarian granulosa cells [31]. Some studies have reported that ADAMTS-1 is induced in granulosa cells in preovulatory follicles after LH administration [32] and is important for follicular development and the maintenance of normal granulosa cell layers in follicles [33]. Endothelin-2 (Edn2), a potent vasoconstrictive peptide, is abundantly produced by preovulatory follicles during ovulation at the onset of CL formation [34]. Edn2 directly induces vascular endothelial growth factor in granulosa cells of the bovine ovary [35] and ovulation and CL formation are significantly impaired in Edn2-knockout mice [34].

Oviduct-specific glycoprotein (OVGP1), also known as oviductin, is the major non-serum glycoprotein in the oviduct fluid during fertilization and increases the number of fertilized eggs and promotes early embryonic development [36]. Furthermore, Edn2 and OVGP1 are primarily localized in the ovaries after native eCG administration. These results suggest that 17 $\beta$ -HSD1, ADAMTS-1, Edn2, and OVGP1 perform pivotal functions as ovulatory factors during ovulation in mice.

## Conclusions

This study shows that rec-eCG produced from CHO-S cells has high biological activity *in vivo*. Although the MCR of rec-eCG is low soon after its administration, rec-eCG displays a wide range of biological activity including the induction of ovulation and oogenesis. The present results show that 63 ovarian genes were differentially expressed between native eCG-treated and rec-eCG-treated ovaries. Differential expression patterns of these genes were further confirmed through RT-PCR, qRT-PCR, and immunostaining analyses. The present results suggest that these differences may have resulted from the nature of the hormone, including oligosaccharides and folding. Further systematic analyses are required to investigate the role of these DEGs in ovulation. Therefore, rec-eCG derivatives can potentially be produced at high levels with high biological activity to induce oocytes *in vivo*.

# Methods

## Materials

The oligonucleotides used herein were synthesized by Genotech (Daejeon, Korea). The restriction enzymes and the DNA ligation kit were purchased from Takara (Tokyo, Japan). The QIAprep-Spin plasmid kit was acquired from QIAGEN, Inc. (Hilden, Germany). The Lumi-Light western blot kit was purchased from Roche (Basel, Switzerland), and the pcDNA3 mammalian expression vector, FreeStyle CHO-S suspension cells, PNGase F, FreeStyle MAX transfection reagent, and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). The PMSG ELISA kit was purchased from DRG International, Inc. (Mountain side, NJ, USA), Centriplus Centrifugal Filter Devices from Amicon Bio separations (Merck, Billerica, MA, USA), and an anti-*myc* antibody and antibodies against HSD17b1, ADAMTS1, Edn2, and OVGP1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Disposable spinner flasks were obtained from Corning Inc. (Corning, NY, USA). A peroxidase-conjugated anti-mouse IgG antibody was obtained from Bio-Rad (Hercules, CA, USA), whereas pregnant-mare serum gonadotropin (eCG) and hCG from Sigma-Aldrich Corp. (St. Louis, MO, USA), as were all other reagents. All protocols complied with the approved Guidelines for Animal Experiments of Hankyong National University, Korea, and were approved by the Animal Care and Use Committee of Hankyong National University, Korea (Approval ID: 2015-8).

## Construction of tethered eCG

cDNA encoding the tethered rec-eCG $\beta/\alpha$  was inserted into the mammalian expression vector pcDNA3, as previously reported [6]. The same method was used to insert a *myc* tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) between the first and second amino acid residues of the  $\beta$ -subunit of the mature eCG protein [27]. Plasmid DNA was then purified and sequenced in both directions through automated DNA sequencing to ensure correct inserts. The cloned expression vector of tethered eCG $\beta/\alpha$  was designated as pcDNA3-eCG $\beta/\alpha$ , as previously reported [6]. A schematic representation for tethered eCG  $\beta/\alpha$  is shown in Fig. 1.

## Cell culture and generation of tethered rec-eCG

In CHO-S cells, the tethered eCG expression vector was transfected into CHO-S cells using the FreeStyle MAX reagent (Invitrogen; Carlsbad, CA, USA) transfection method, in accordance with manufacturer's instructions. Flasks were placed on an orbital shaking platform, rotating at 120–135 rpm at 37°C in a humidified atmosphere of 8% CO<sub>2</sub> in air. On transfection, the cell density was approximately 1.2–1.5 × 10<sup>6</sup> cells/mL. The plasmid DNA (260 ug) and a FreeStyle™ MAX Reagent complexes were gradually added to 200 mL of medium containing cells. Finally, culture media were sampled on day 9 after transfection and centrifuged to eliminate cell debris. The supernatant was sampled and stored at -20°C until the assay. The samples were concentrated using a Centricon filter or by freeze-drying and mixed with PBS.

## Quantification of rec-eCG proteins

rec-eCG protein was quantified with the PMSG ELISA kit (DRG Diagnostics; Mountain side, NJ, USA). Briefly, the PMSG standard and rec-eCG samples (100  $\mu$ L) were dispensed into the wells of a plate coated with the antibody and incubated for 60 min at ambient temperature. After rinsing thrice, 100  $\mu$ L of anti-PMSG antibody conjugated with horseradish peroxidase was added into each well and incubated for 60 min. The plate wells were rinsed five times, and substrate solution (100  $\mu$ L) was added and incubated for 30 min at ambient temperature. Finally, 50  $\mu$ L of a stop solution was added and the absorbance was measured at 450 nm, using a microtiter plate reader Cytation<sup>TM</sup> 3 (BioTeK, Winooski, VT, USA). The average absorbance of each standard was plotted against its corresponding concentration in a linear-log graph. We determined the average absorbance of each sample to determine the corresponding PMSG value via simple interpolation through a standard curve. Finally, 1 IU was considered 100 ng in accordance with the conversion factor of the suggested assay protocol.

### **Detection of rec-eCGs via western blotting and enzymatic digestion of N-linked oligosaccharides**

Concentrated sample media were subjected to SDS-PAGE (12.5% resolving gel) via the Laemmli method [37]. After SDS-PAGE, the proteins were electro-transferred to a nitrocellulose membrane for 2 h in a Mini Trans-Blot Electrophoretic Transfer cell. To eliminate all N-linked oligosaccharides, the rec-eCG sample was incubated for 24 h at 37°C with PNGase F [2 mL of the enzyme (2.5 U/mL) per 30 mL of sample+8 mL of 5' reaction buffer]. The reaction was terminated by boiling for 10 min, and the samples were subjected to SDS-PAGE and the proteins were electro-transferred on to a membrane. After blocking the membrane with a 1% blocking reagent for 1 h, followed by probing with monoclonal anti-*myc* antibody (1:5,000) for 2 h, the membrane was washed and probed with a secondary antibody (peroxidase-conjugated anti-mouse IgG antibody 37.5  $\mu$ L/15 mL of the blocking solution) for 30 min. The membrane was then incubated for 5 min with 2 mL of the Lumi-Light substrate solution and X- ray film was exposed to the membrane for 1–10 min.

### **Assessment of the MCR of eCGs**

Each animal was intravenously administered 5 IU of native eCG or rec-eCG through the tail vein to determine the 50% dose for the induction of superovulation. Blood was sampled from the transorbital vein in heparinized microhematocrit tubes. Blood samples were obtained at 10 and 30 min and at 1, 2, and 24 h and centrifuged for 15 min at 5,000 rpm at 4°C, and plasma eCG concentrations were estimated using the PMSG ELISA kit (DRG Diagnostics).

### **Animals**

The MCRs of native eCG and rec-eCG were determined in 8-week-old male B6D2F1 (C57BL6  $\times$  DBA/2) 12 mice. The female 16 mice (8-week-old B6D2F1; Oriental Bio, Gyeonggi, Korea) were superovulated by injection of 10 IU of native eCG and rec-eCG and then 10 IU hCG after 48 h. The ovarian tissues were sampled at 13 h after hCG administration. All mice were euthanized with carbon dioxide inhalation, and the ovarian tissues were collected at the end of study. All the mice were raised in an environment with the temperature of 23  $\pm$  1 °C with regular 12 h light/dark cycle and allowed free access to feed and water. The

animals were processed by the Animal Care and Use Committee procedure. The protocol was approved by the Committee on Ethics of Animal Experiments at the Hankyong National University (Approval ID: 2015-8).

## Microarray analysis

Total RNA was extracted from ovaries, using TRIzol reagent, and purified using RNeasy columns in accordance with the manufacturers' protocols, as previously described [15].

### 1) Labeling and purification

Total RNA was amplified and purified using an Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions to obtain biotinylated cRNA. Briefly, 550 ng of total RNA was reverse-transcribed into cDNA with a T7 oligo(dT) primer. Second-strand cDNA was synthesized, transcribed *in vitro*, and labeled with biotin-NTP.

### 2) Hybridization and data export

Labeled cRNA samples (0.75 µg) were hybridized to the Illumina MouseRef-8 v2 expression BeadChip (Illumina, Inc., San Diego, CA, USA) for 16–18 h at 58°C. Array signals were detected using Amersham Fluorolink Streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) in accordance with the manufacturer's instructions. Arrays were scanned using an Illumina bead array reader (confocal scanner). Array data were analyzed using in Illumina Genome Studio v.2009.2 software (Gene Expression Module v.1.5.4).

### 3) Raw data preparation and statistical analysis

Raw data were extracted using the software provided by the manufacturer (Illumina Genome Studio v.2009.2) and filtered using a detection p-value of <0.05 (a signal value higher than that of the background was necessary to set the detection p-value of <0.05). The selected gene signal value was logarithmically transformed and normalized to XYZ. Comparative analysis between two groups was conducted on the basis of the p-value evaluation, via the local-pooled-error test (adjusted Benjamini–Hochberg false discovery rate had to be <5%) and the fold-change. Biological ontology-based analysis was performed for the Panther database (<http://www.pantherdb.org>). Furthermore, genes whose expression levels differed by >2-fold were considered differentially expressed between the two groups.

## RT-PCR and qRT-PCR analyses

To validate the microarray data, 14 genes (up-regulated: *Tex19.2*, *Sectm1b*, *Ctsk*, *Gpnmb*, *Sectm1a*, and *Hsd17b1*; down-regulated: *OVGP1*, *BC048546*, *Tmem68*, *Dcpp1*, *Prkg2*, *Edn2*, *Adamts1*, and *Akr1b7*) from different groups were evaluated through RT-PCR and qRT-PCR analysis, their expression levels differed by >2-fold. RT-PCR and qRT-PCR analysis was performed for the same ovarian tissue subjected to microarray analyses. Primer sequences are outlined in Supplementary Material Table 1 along with the primer

annealing temperatures. The primers were designed using Primer3 software (<http://www.bioneer.co.kr/tools/>). Ovarian gene expression levels were then normalized to those of *Actb* via the  $2^{-\Delta\Delta CT}$  method for quantitative relation.

## Immunohistochemistry

Immunohistochemical staining of ovarian samples was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) in accordance with the manufacturer's instructions. The samples were fixed in 10% neutral-buffered formalin at ambient temperature for 24 h and washed with PBS. Thereafter, the fixed samples were rehydrated in graded ethanol (EtOH) solutions (3 min each in 100% 2'; 95% 1'; 70% 1'; and 50% 1') and embedded in paraffin. Paraffin-embedded tissues were sectioned into 8- $\mu$ m-thick sections, which were then 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. Thereafter, they were washed with 3% hydrogen peroxide for 10 min and blocked for 1 h at ambient temperature. The slides were incubated with the primary antibody and then with an anti-rabbit IgG antibody (secondary antibody). Finally, the slides were immunostained using the ABC detection kit in accordance with the manufacturer's instructions and stained with DAB. The slides were examined under a Nikon Eclipse TE-2000-E confocal microscope (Tokyo, Japan).

## Data and statistical analysis

Data are presented as mean  $\pm$  SEM values. One-way ANOVA with Tukey's multiple-comparison test was conducted to compare the results between samples. In figures, the superscripts indicate significant differences from groups ( $p < 0.05$ ).

## Abbreviations

rec-eCG: recombinant equine chorionic gonadotropin, LH: luteinizing hormone, FSH: follicle-stimulating hormone, TSH: thyroid-stimulating hormone, FSHR: FSH receptor, PMSG: pregnant mare serum gonadotropin, MCR: metabolic clearance rate, qRT-PCR: quantitative reverse-transcription-polymerase chain reaction, HSD: hydroxysteroid dehydrogenase, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Edn2: endothelin-2, OPGP1: oviduct-specific glycoprotein1

## Declarations

### Ethics approval and consent to participate

All experimental designs and procedures complied with the approved Guidelines for Animal Experiments of Hankyong National University, Korea and were approved by the Animal Care and Use Committee of Hankyong National University, Korea (Approval ID: 2015-8).

### Consent for publication

Not applicable.

## Availability of data and material

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

JJP conducted the experiments. MB performed ELISA and interpreted the data. LSY prepared the figures and supplementary file. MHK and KSM wrote the manuscript. All authors reviewed the final manuscript.

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## Tables

**Table 1.** Genes that were up-regulated (fold-change) in rec-eCG-treated ovaries.

No.	Symbol	Accession No.	Fold rec-eCG/native eCG
1	Ercc5	NM_011729.1	2.04
2	Hexdc	NM_001001333.1	2.05
3	Arfgap2	NM_023854.1	2.16
4	Rbm35a	NM_194055.1	2.20
5	LOC100046802	XM_001476835.1	2.30
6	S100a13	NM_009113.3	2.46
<b>7</b>	<b>Hsd17b1</b>	<b>NM_010475.1</b>	<b>2.48</b>
8	Prcp	NM_028243.2	2.49
9	Cyp19a1	NM_007810.2	2.50
10	BC010462	NM_145373.1	2.62
<b>11</b>	<b>Sectm1a</b>	<b>NM_145373.2</b>	<b>2.64</b>
<b>12</b>	<b>Gpnmb</b>	<b>NM_053110.3</b>	<b>2.82</b>
13	Csrp1	NM_007791.4	2.88
14	C130060K24Rik	NM_175524.3	2.97
<b>15</b>	<b>Ctsk</b>	<b>NM_007802.3</b>	<b>3.11</b>
16	Crybb1	NM_023695.2	3.17
17	Rbm8a	NM_025875.1	3.22
<b>18</b>	<b>Sectm1b</b>	<b>NM_026907.3</b>	<b>3.47</b>
<b>19</b>	<b>Tex19.2</b>	<b>NM_027622.2</b>	<b>3.57</b>
20	Rpusd4	NM_028040.2	3.80

Bold genes were adjusted to RT-PCR and qRT-PCR

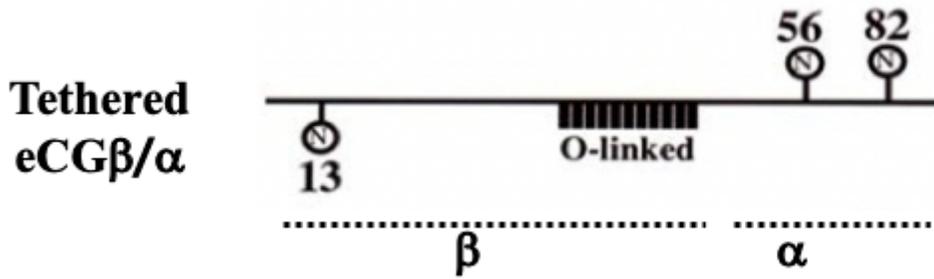
**Table 2.** Genes that were down-regulated (fold-change) in rec-eCG-treated ovaries.

No.	Symbol	Accession No.	Fold Rec-eCG/native eCG
1	<b>Ovgp1</b>	<b>NM_007696.2</b>	<b>-18.31</b>
2	<b>BC048546</b>	<b>NM_001001179.2</b>	<b>-10.61</b>
3	BC048546	NM_001001179.1	-10.28
4	Emb	NM_010330.3	-6.64
5	<b>Tmem68</b>	<b>NM_028097.3</b>	<b>-5.81</b>
6	<b>Dcpp1</b>	<b>NM_019910.2</b>	<b>-5.10</b>
7	Ltf	NM_008522.3	-4.97
8	1500015010Rik	NM_024283.2	-3.09
9	Dynlrb2	NM_029297.1	-2.97
10	Il1rn	NM_031167.3	-2.89
11	<b>Prkg2</b>	<b>NM_008926.3</b>	<b>-2.81</b>
12	Mmp10	NM_019471.2	-2.78
13	Spag1	NM_012031.1	-2.77
14	<b>Edn2</b>	<b>NM_007902.2</b>	<b>-2.73</b>
15	Tnfrsf11b	NM_008764.3	-2.62
16	Cops8	NM_133805.3	-2.55
17	Trib3	NM_175093.2	-2.53
18	EG240916	NM_177723.2	-2.51
19	Cln5	XM_127882.3	-2.49
20	Bves	NM_024285	-2.46
21	Spp1	NM_009263.1	-2.43
22	Adcyap1	NM_009625.2	-2.36

No.	Symbol	Accession No.	Fold Rec-eCG/native eCG
23	Dmbt1	NM_007769.1	-2.35
24	Fndc7	NM_177091.2	-2.33
25	Rhox8	NM_001004193.2	-2.32
26	2600011E07Rik	NM_028113.1	-2.24
<b>27</b>	<b>Adamts1</b>	<b>NM_009621.3</b>	<b>-2.24</b>
28	Serpinb2	NM_011111.3	-2.21
29	Dnali1	NM_175223.2	-2.20
30	Abhd2	NM_018811.6	-2.19
31	Kndc1	NM_177261.4	-2.19
32	Xpnpep2	NM_133213.2	-2.16
33	Abhd2	NM_018811.6	-2.15
34	Ier3	NM_133662.2	-2.13
35	Ankrd1	NM_013468.2	-2.12
<b>36</b>	<b>Akr1b7</b>	<b>NM_009731.1</b>	<b>-2.09</b>
37	Serpina3n	NM_009252.2	-2.09
38	V1rd6	NM_030738.1	-2.08
39	Gdpd3	NM_024228.2	-2.08
40	1110049B09Rik	NM_001024478.1	-2.05
41	Aldh1l2	NM_153543.1	-2.02
42	Cd177	NM_026862.3	-2.02
43	2010001J22Rik	NM_001013022.1	-2.00

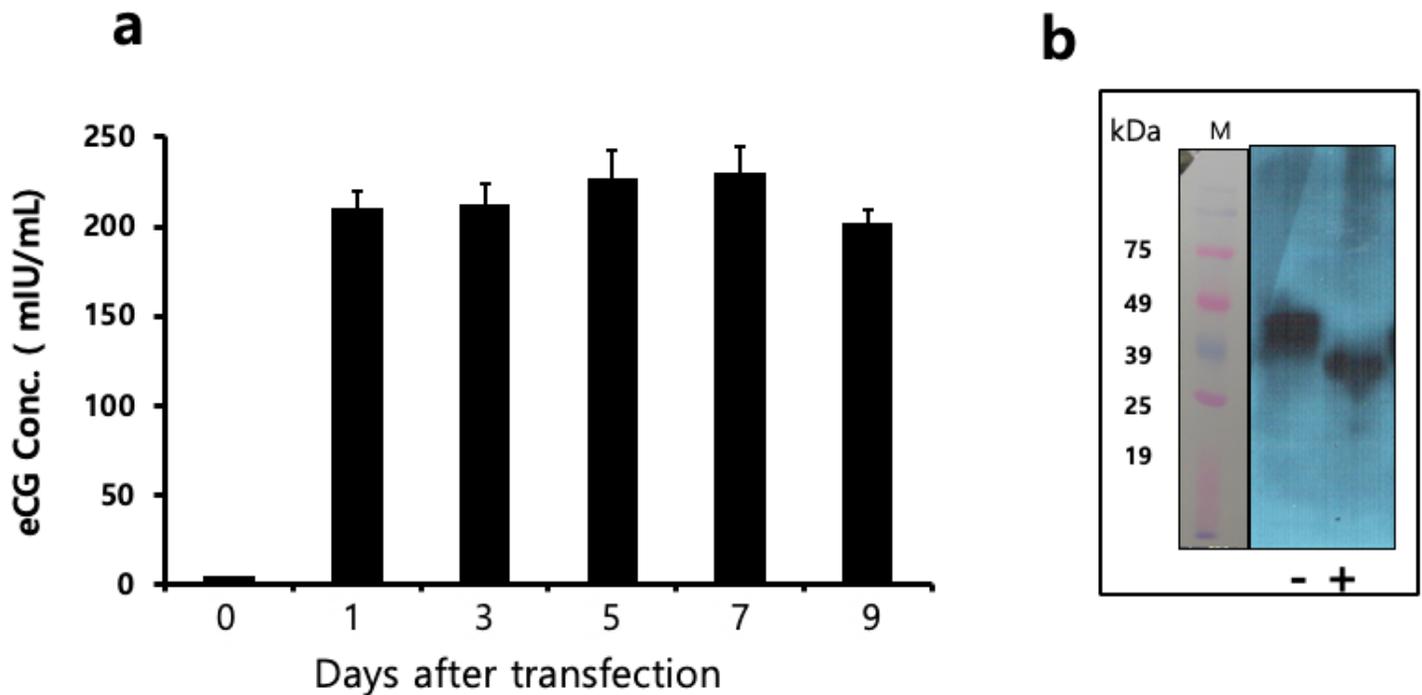
Bold genes were adjusted to RT-PCR and qRT-PCR

## Figures



**Figure 1**

A schematic representation of tethered rec-eCG. The wild-type protein with N- and O-linked oligosaccharide sites on eCG are shown. The circle “N” denotes an N-linked oligosaccharide. The plasmids encoding tethered rec-eCG are designated as pcDNA3-eCG.



**Figure 2**

Quantitative analysis and western blot analysis of tethered rec-eCG. A) Quantities of myc-tagged rec-eCG mutants were analyzed by an ELISA. The myc-tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was inserted between the first and second amino acid residue of the  $\beta$ -subunit of mature eCG protein. B) Western blot analysis of tethered rec-eCG. The proteins in conditioned media were separated via SDS-PAGE and electro-transferred to a blotting membrane. The proteins were detected with antibodies against the myc-tag. Proteins for western blotting were also treated with N-Glycosidase F. -: not treated, +: treated with N-Glycosidase-F.

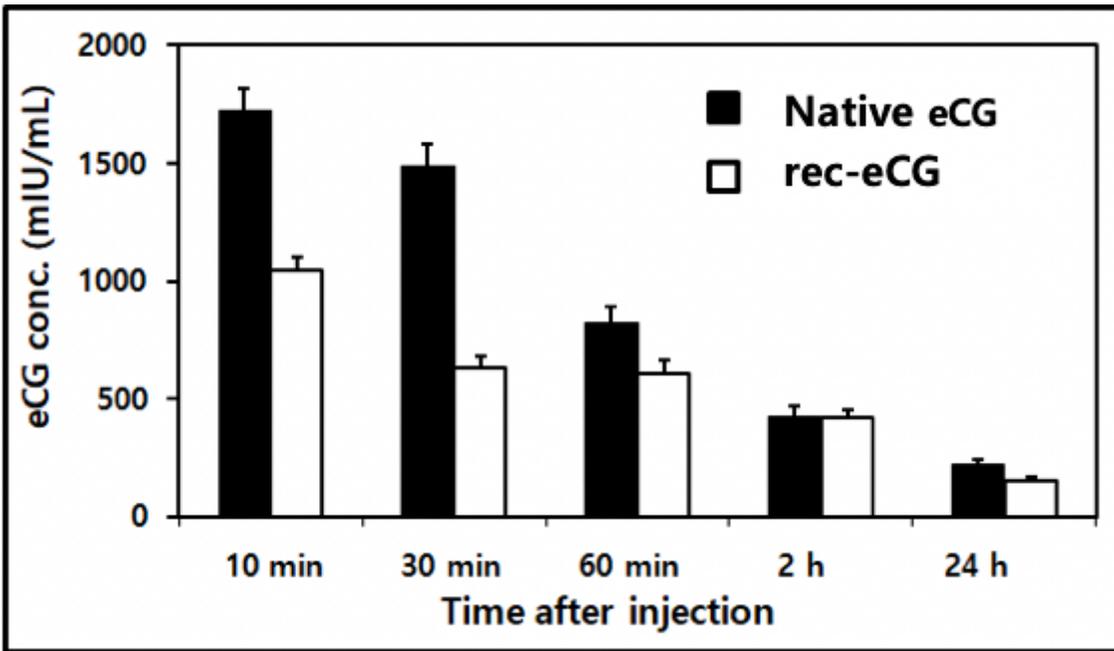
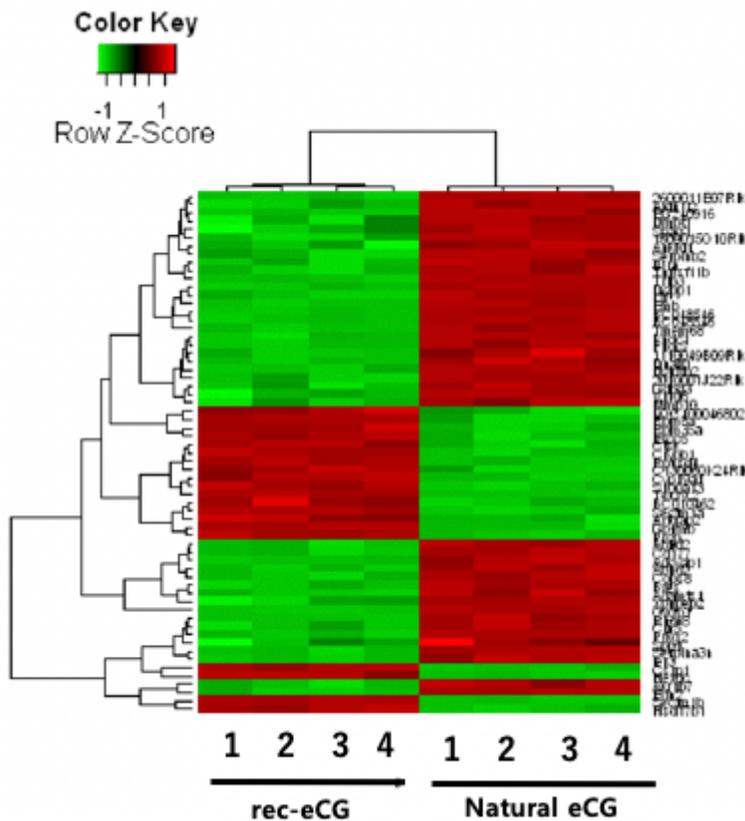


Figure 3

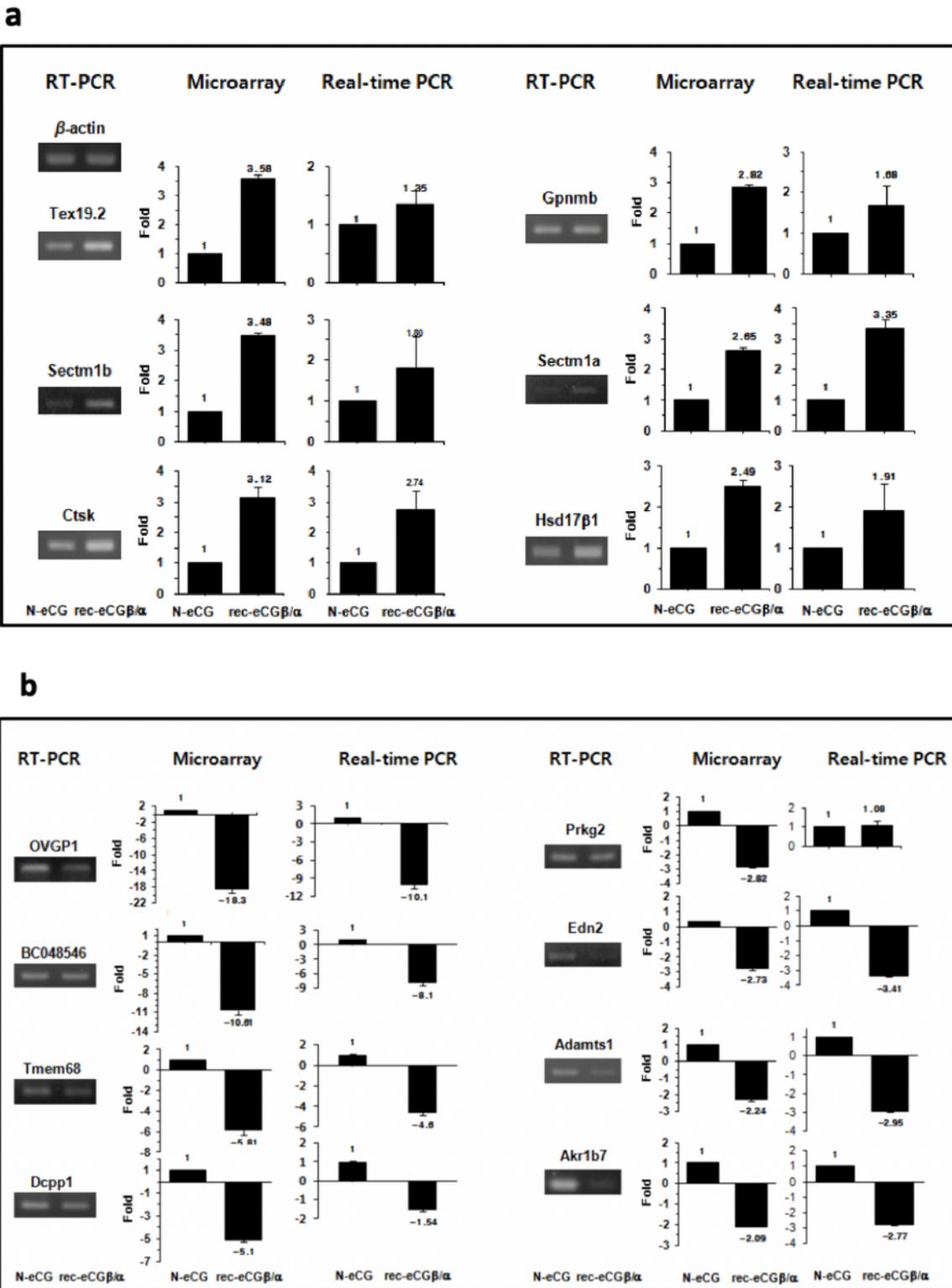
The metabolic clearance rate (MCR) of native eCG and of rec-eCG. Both eCGs were intravenously administrated at 5 IU through the tail vein. Blood samples were collected after 10 and 30 min and 1, 2, and 24 h. The samples were centrifuged at 5,000 rpm for 15 min at 4°C, and eCG concentrations in the serum were estimated using a PMSG ELISA kit. The levels of eCG were analyzed via sandwich ELISA in triplicate.



Category		# of DEGs
rec-eCG vs Native eCG	UP	20
	DOWN	43
Total(UP+DOWN):		63

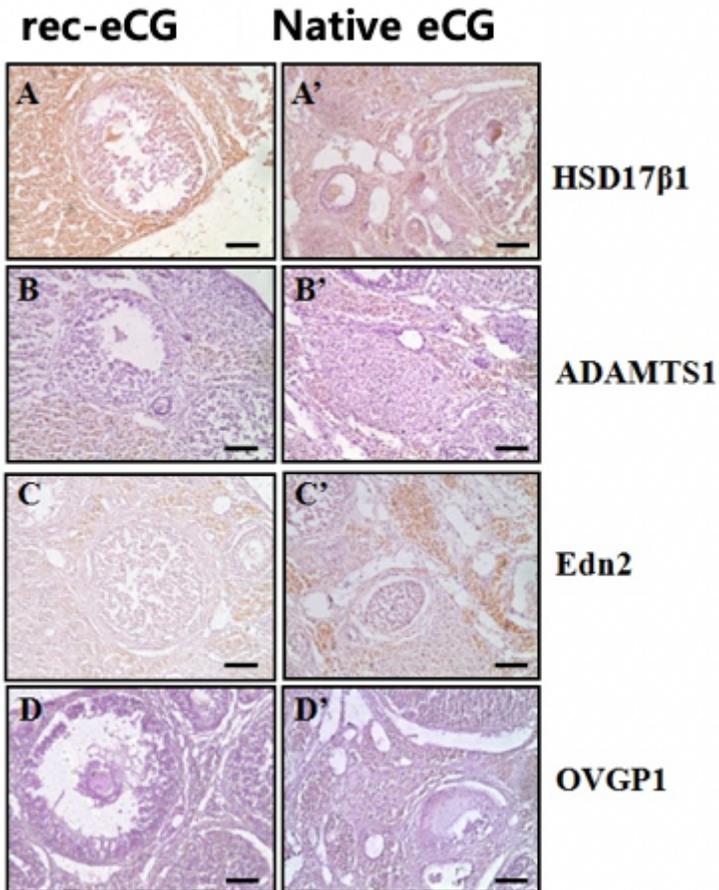
## Figure 4

Hierarchical clustering of gene expression profiles in native eCG-treated and rec-eCG-treated ovarian tissues. The ovaries were excised from 8-week-old ICR female mice. The mice were induced to superovulate with 10 IU of native eCG or rec-eCG and then 10 IU of hCG after 48 h, and the ovulated oocytes were collected in an oviduct ampulla after 13 h. Thereafter, the ovaries were harvested after 13h and RNA was analyzed via microarray analysis. Gene expression levels were evaluated through microarray analysis with 12,816 gene probes. Genes showing >2-fold differences in expression were identified. The expression of 63 (0.49%) of 12,816 genes differed by at least 2-fold between native eCG-treated and rec-eCG-treated ovaries. Twenty of 63 genes were up-regulated in rec-eCG-treated ovaries and 43 genes were down-regulated



**Figure 5**

Quantitative real-time PCR (qRT-PCR) and reverse-transcription PCR (RT-PCR) analyses. (a) Fourteen genes from different categories were analyzed via qRT-PCR. Six genes were up-regulated in the rec-eCG-treated ovaries. (b) The other eight genes were down-regulated in rec-eCG-treated ovaries. The microarray results were compared and further analyzed via RT-PCR and qRT-PCR. Actb served as an endogenous control.



**Figure 6**

Localization of HSD17 $\beta$ 1, ADAMTS1, EDN2, and OVGP1. The ovaries were induced to superovulate with 10 IU of either natural-eCG or rec-eCG, followed by 10 IU of hCG after 48 h. Representative immunohistochemical analyses for HSD17 $\beta$ 1, ADAMTS1, EDN2, and OVGP1 were conducted with antisera, and a goat anti-rabbit IgG antibody (secondary antibody). According to the microarray and qRT-PCR results, HSD17 $\beta$ 1 was up-regulated in the rec-eCG-treated ovaries, while the other three proteins (ADAMTS1, EDN2, and OVGP1) were up-regulated in the native eCG-treated ovaries. Immunohistochemistry was performed with a Vectastain ABC kit. Scale bar = 200  $\mu$ m.