

—Technology Report—

## Construction and expression of vectors encoding biologically active rodent gonadotropins

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**Abstract.** The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are important hormones in vertebrate reproduction. The isolation of gonadotropins from the pituitary gland is sub-optimal, as the cross-contamination of one hormone with another is common and often results in the variation in the measured activity of LH and FSH. The production of recombinant hormones is, therefore, a viable approach to solve this problem. This study aimed to express recombinant rat, mouse, and mastomys FSH and LH in Chinese hamster ovary (CHO) cells. Their common  $\alpha$ -subunits along with their hormone-specific  $\beta$ -subunits were encoded in a single mammalian expression vector. FSH from all three species was expressed, whereas expression was achieved only for the mouse LH. Immunohistochemistry for rat alpha subunit of glycoprotein hormone ( $\alpha$ GSU) and LH $\beta$  and FSH $\beta$  subunits confirmed the production of the dimeric hormone in CHO cells. The recombinant rodent gonadotropins were confirmed to be biologically active; estradiol production was increased by recombinant FSH in granulosa cells, while recombinant LH increased testosterone production in Leydig cells.

**Key words:** Follicle-stimulating hormone, Luteinizing hormone, Mastomys, Pituitary, Recombinant

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**F**ollicle-stimulating hormone (FSH) and luteinizing hormone (LH), synthesized in the anterior lobe of the pituitary gland, cooperatively modulate the cycle of the ovarian follicle (maturation, ovulation, and luteinization), accounting for different phases of the reproductive cycle [1–4]. The follicular growth in the female ovary requires FSH, while LH induces ovulation from mature follicles and maintains progesterone production by the corpus luteum. In the male testis, FSH supports Sertoli cells to induce the growth of the seminiferous tubules and to maintain spermatogenesis, while LH stimulates androgen synthesis in the Leydig cells. To study FSH and LH biology, preparations of these molecules are required to be pure, as contamination with each other or other hormones may produce unexpected results. In this respect, recombinant hormones are guaranteed to be free from any other hormonal contamination.

While performing experiments with hormones, it is preferable to use the appropriate hormone derived from the same species rather than from a different species [5]; however, the production of recombinant gonadotropins from different species is challenging [6–8]. The successful production of bioactive gonadotropins has been

unattainable in either bacteria or yeast, as these cells are unable to perform appropriate glycosylation, a modification that is important for the biological activity of gonadotropins, of recombinant hormones. In this study, we constructed mammalian expression vectors encoding gonadotropins from three different rodent species —rat, mouse, and mastomys (*Praomys coucha*), a rodent endemic to Africa— and used them for the production of bioactive hormones in Chinese hamster ovary (CHO) cells. Mastomys was first used as a laboratory animal about 60 years ago. As an experimental animal, mastomys has several attractive features. It is intermediate in size between mouse and rat, easy to breed, produces large litters, and spontaneously ovulates all year round. For these reasons, this animal is widely used in the field of reproductive biology, particularly as a source of pre-implantation embryos for *in vitro* culture, as well as for superovulation studies [9, 10]. The present study accordingly aimed to produce recombinant (rec)FSH and recLH from these three rodent species to support future reproductive biology research.

We constructed expression vectors containing dual promoters to express both the alpha subunit of glycoprotein hormone ( $\alpha$ GSU) and  $\beta$  subunits of FSH and LH in the same transfected mammalian cell. For the construction of the rat and mastomys FSH expression vectors, the rat and mastomys  $\alpha$ GSU and FSH $\beta$  cDNAs were excised from the pGEM-T easy vector and inserted downstream of the CAG promoter in pCX vector (Fig. 1). The rat or mastomys CAG- $\alpha$ GSU cDNA fragments were then excised and the cut ends were blunted and inserted into *HincII* site of the respective species pCX-FSH $\beta$  vectors. The puromycin resistance gene was inserted into both of these vectors to allow for the selection of stably transformed cell lines, resulting in

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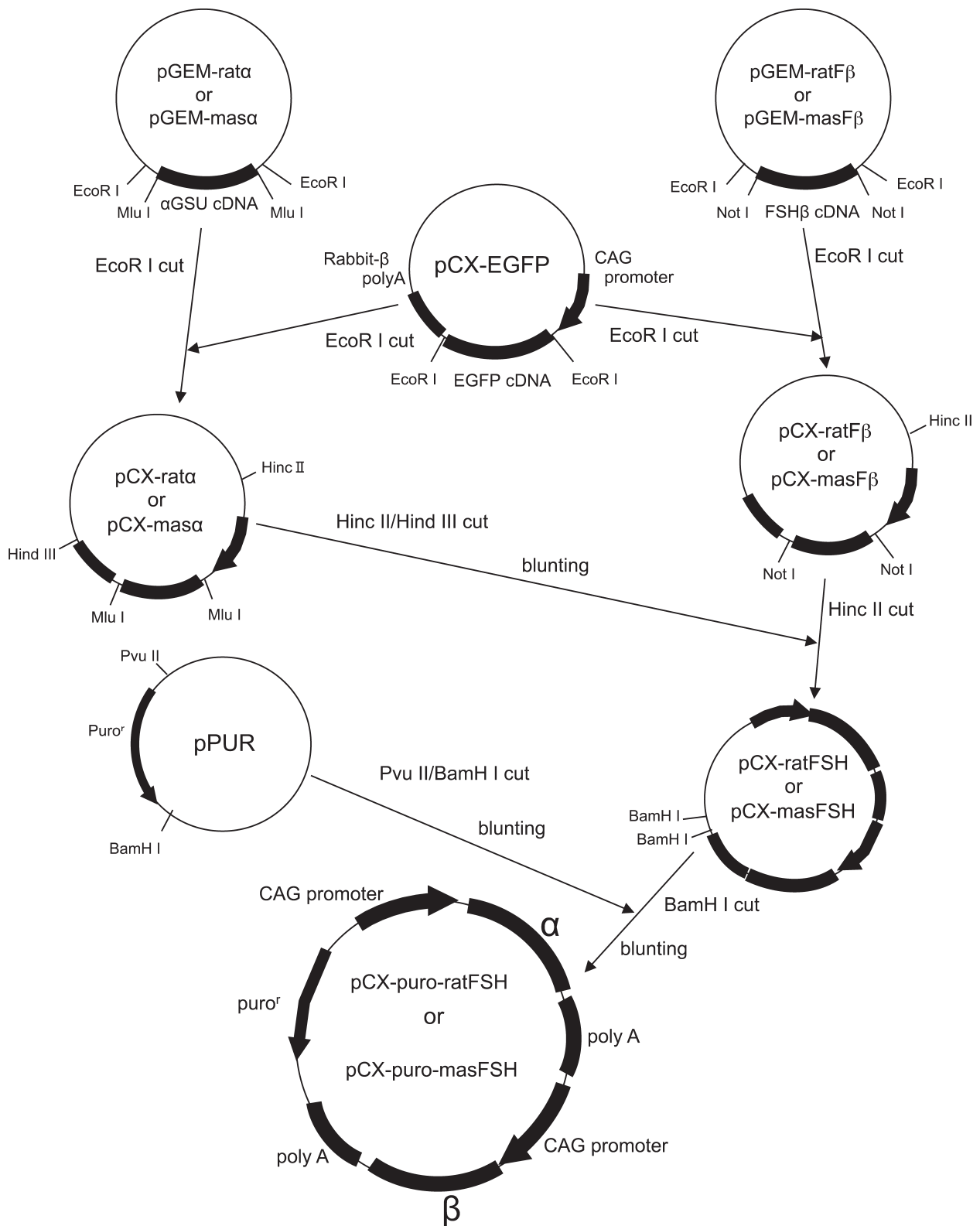
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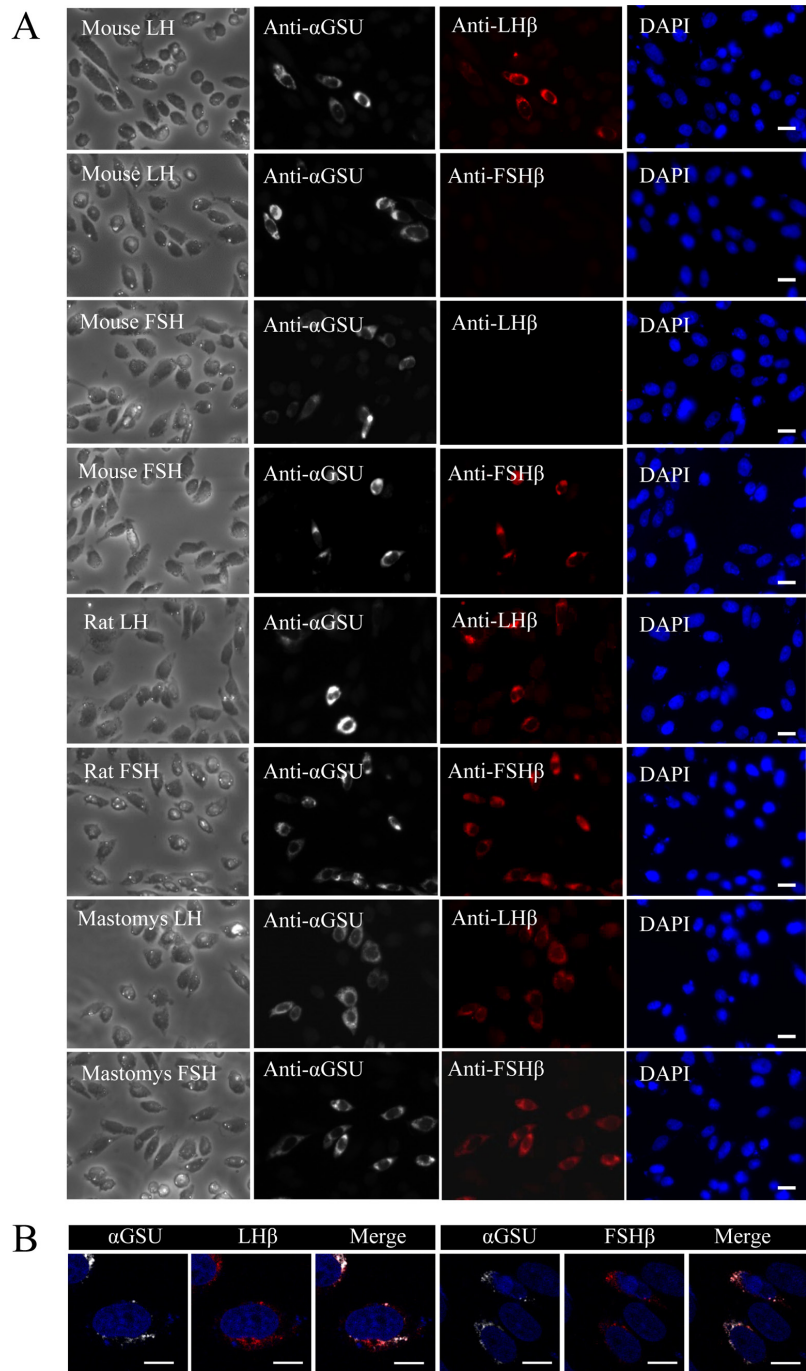
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**Fig. 1.** Procedure used for the construction of FSH expression vector. CAG: CMV immediate early enhancer element, the chicken  $\beta$ -actin promoter with the first exon and first intron of the chicken beta-actin gene, and the splice acceptor from rabbit  $\beta$ -globin. EGFP cDNA: enhanced green fluorescent protein cDNA. poly A: rabbit  $\beta$ -globin polyadenylation signals.  $\alpha$ GSU cDNA: rat or mastomys (mas)  $\alpha$ GSU-coding region. FSH $\beta$  cDNA: rat or mastomys (mas) FSH $\beta$ -coding region. Puro<sup>r</sup>: puromycin resistance gene.



**Fig. 2.** Transfection of expression vectors encoding rodent gonadotropins. Transfection of six expression vectors constructed in this study was performed using Chinese hamster ovary (CHO) cell line cultured in eight-well chambered slides. The cells were cultured for 24 h and immunostained with guinea pig antiserum against rat  $\alpha$ GSU (Cy3, white), LH $\beta$  (Cy5, red), and FSH $\beta$  (Cy5, red) subunits. (A) Bright field images are shown in the far left panel and fluorescence microscopy images are shown in the other three panels. (B) Confocal microscopy images. Immunocytochemistry data are shown only for mouse  $\alpha$ GSU and either LH $\beta$  or FSH $\beta$  expression. Bar = 10  $\mu$ m.

the generation of pCX-puro-ratFSH (encoding rat recFSH) and pCX-puro-masFSH (encoding mastomys recFSH). Other expression vectors encoding recombinant mouse FSH as well as recombinant mouse, rat, and mastomys LHs were constructed by replacing  $\alpha$ GSU, FSH $\beta$ ,

or LH $\beta$  cDNA with the respective cDNAs using pCX-puro-masFSH. Further details concerning these expression vectors are provided in the Supplementary Tables and Figures (online only).

The goal of this study was to produce and examine the bioactivity

of recFSH and recLH from rat, mouse, and mastomys. However, we failed to accomplish this goal for recLH from rat and mastomys. Nonetheless, we have provided details of all the clones and their full cDNA sequence information for further studies using alternative strategies. All experiments were conducted using conditioned media from the recombinant stable CHO lines expressing gonadotropins. After the generation of puromycin-resistant cell lines, the expression of  $\alpha$ GSU, FSH $\beta$ , and LH $\beta$  was examined by reverse transcription polymerase chain reaction (RT-PCR; Supplementary Fig. 1), followed by the analysis of the levels of FSH and LH secreted into the culture medium (Supplementary Table 1). As differences were reported in the gonadotropin yields, cell lines with higher yields were selected for further analyses. A time-resolved fluorescence immunoassay (TR-FIA) confirmed the appropriate antigenicity for all three recFSHs and mouse recLH (Supplementary Fig. 2), indicative of the formation of the appropriate  $\alpha/\beta$  dimers. In addition, TR-FIA analysis showed that CHO cells displayed varying expression of recombinant gonadotropins, ranging from 38 to 1,236 ng/ml culture medium over a 24-h period depending on the cell line, as shown in Supplementary Table 1. The mass production of rat and mastomys recLHs was not examined. Although the yields reported herein are lower than those observed with a baculovirus expression system expressing porcine FSH [11], the production of gonadotropins in CHO cells allows for normal glycosylation, which is crucial for the biological activities of gonadotropins. We also examined the production of mastomys recFSH using an internal ribosome entry vector (pIRES, BD Bioscience Clontech, Palo Alto, CA, USA), which resulted in the production of low levels of mastomys recFSH (9–16 ng/ml over 24 h).

Immunohistochemistry was performed in CHO cells expressing rat, mouse, or mastomys gonadotropins. After 48 h of culture, cells were fixed and probed with antibodies against  $\alpha$ GSU, LH $\beta$ , or FSH $\beta$ . Positive signals for  $\alpha$ GSU, LH $\beta$ , or FSH $\beta$  were seen in CHO cells expressing the constructs from mouse (Fig. 2B, first and fourth rows), rat (Fig. 2A, fifth and sixth rows), and mastomys (Fig. 2A, seventh and eighth rows). The absence of any cross-reaction between anti-LH $\beta$  antibody and FSH $\beta$  subunit as well as anti-FSH $\beta$  antibody and LH $\beta$  subunit was confirmed (Fig. 2A, second and third rows). The data show that the two gonadotropin subunits could be successfully synthesized in the same cell, although the quantity of both subunits was not determined.

The bioactivities of the recombinant gonadotropins, namely rat, mouse, and mastomys FSHs and mouse LH were examined. As shown in Supplementary Fig. 3, granulosa cell aromatase bioassay revealed a dose-dependent increase in the production of estradiol with rat, mouse, and mastomys recFSHs. The bioactivity of mouse recLH was assessed using rat Leydig cells. Mouse recLH was able to increase the testosterone production, although its dose response differed to that of human chorionic gonadotropin (hCG; Supplementary Fig. 4).

We performed the superovulation assay using mastomys and mouse recFSHs and found that the recovery of ova from mastomys and mice treated with a combination of recFSH and hCG was significantly higher than that in animals treated with hCG alone (Supplementary Table 2). In addition, ovary weight was significantly increased in mastomys treated with recFSH and hCG (Supplementary Table 2). After superovulation, the ovaries of young mastomys were examined by histology. As shown in Supplementary Fig. 5, the number of

ova, especially ovulatory follicles, increased in animals treated with recFSH and hCG as compared with those treated with hCG alone.

Seminal vesicle weight gain (SVW) assays using recLH were performed (Supplementary Table 3). Although only a small number of animals were analyzed, the treatment with mouse recLH produced a similar but significant increase in seminal weight, as observed with hCG treatment.

With the exception of rat and mastomys LHs, this study reports the successful production of biologically active, rat, mouse, and mastomys recFSHs as well as mouse recLH. Therefore, these vectors may display potential applications in reproductive research.

Immunocytochemical analyses revealed a granular distribution of the recombinant hormones. We analyzed these hormones using confocal laser scanning microscopy. As shown in Fig. 2B, the signals for the hormone subunits were co-localized in the cytoplasm. Beuret *et al.* reported that CHO cells have the ability to form constitutive secretory granules [12]; hence, recombinant gonadotropins may be stored within these granules. However, the fact that recombinant, bioactive hormones could be recovered from the culture medium suggests the presence of a constitutive or stimulus-secretion mechanism for the release of these hormones in CHO cells.

## Methods

### *Construction of expression vectors*

Total RNAs from the pituitary gland of male rat (Wistar-Imamichi strain), mouse (C57BL/6 strain), and mastomys (Jms: CHAM strain) were prepared under the approval of the committee on animal experiments of the School of Agriculture, Meiji University. cDNAs were synthesized using a Superscript first-strand cDNA synthesis for RT-PCR (Invitrogen, Carlsbad, CA, USA) and full-length cDNAs were amplified using the primer sets listed in Supplementary Table 4.

The expression vectors encoding rat and mastomys FSH were constructed according to the following procedure (Fig. 1 and Supplementary Fig. 6). Rat and mastomys  $\alpha$ GSU and FSH $\beta$  cDNAs were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) by TA cloning, resulting in the creation of pGEM-rata, pGEM-masa, pGEM-ratF $\beta$ , and pGEM-masF $\beta$ . These cDNAs were excised from pGEM vector by *EcoRI* digestion and ligated into *EcoRI* site of the mammalian expression vector, pCX-EGFP [13], which was previously digested with *EcoRI* to remove the EGFP cDNA; thus, pCX-rata, pCX-masa, pCX-ratF $\beta$ , and pCX-masF $\beta$  were created. Rata and masa were then excised from pCX-rata and pCX-masa using *HincII/HindIII* double digestion, followed by the creation of blunt ends. These were then ligated to *HincII*-digested pCX-ratF $\beta$  or pCX-masF $\beta$ , thereby creating pCX-ratFSH and pCX-masFSH, respectively. The puromycin resistance gene was obtained by *PuvII/BamHI* digestion from pPUR vector (BD Bioscience Clontech). After creation of the blunt ends, the gene was inserted into *BamHI*-digested/blunted site of pCX-ratFSH and pCX-masFSH, resulting in the creation of pCX-puro-ratFSH and pCX-puro-masFSH, respectively.

The expression vectors encoding mouse LH and FSH were obtained by replacing the sequences encoding FSH in pCX-puro-masFSH. Full-length cDNAs encoding mouse  $\alpha$ -subunit, mouse FSH $\beta$ , and mouse LH $\beta$  were cloned into pGEM-T easy vector using the primer sets shown in Supplementary Table 4, resulting in the production

of pGEM-mou $\alpha$ , pGEM-mouF $\beta$ , and pGEM-mouL $\beta$ , respectively (Supplementary Fig. 7). The mouse  $\alpha$ -subunit was excised by *MulI* digestion and ligated to *MulI*-digested pCX-puro-masFSH, resulting in the production of pCX-puro-mou $\alpha$ /masF $\beta$ . Each mouse F $\beta$  and mouse L $\beta$  fragment was excised by *NotI* digestion and ligated to *NotI*-digested pCX-puro-mou $\alpha$ /masF $\beta$ , resulting in the creation of pCX-puro-mouFSH and pCX-puro-mouLH.

The amino acid sequence of the mastomys  $\alpha$ -subunit is identical to that of the mouse  $\alpha$ -subunit, whereas a single amino acid difference exists between LH $\beta$  subunit of mouse and mastomys. Hence, a single nucleotide mutation was made in pGEM-mouLH $\beta$  to create the sequence of mastomys LH $\beta$  and the product was used to generate pCX-puro-masLH.

The expression vector for rat LH was obtained using pCX-puro-ratFSH. The full-length cDNA encoding rat LH $\beta$  (Supplementary Fig. 8A) was cloned into the pGEM-T easy vector using the primer sets described (Supplementary Table 4), resulting in the generation of pGEM-ratLH $\beta$ , followed by its excision with *NotI* and ligation to the *NotI*-digested pCX-puro-ratFSH to obtain pCX-puro-ratLH.

During all cloning procedures, nucleotide sequencing and restriction enzyme digestion analyses were performed to confirm cDNA insertion and orientation. Supplementary data include the nucleotide sequence information for physical maps of all expression vectors (Supplementary Fig. 8A), each expression vector (Supplementary Fig. 8B–G), amino acid sequences derived from the cDNA-coding regions (Supplementary Fig. 9), and amino acid sequence similarities between each hormone subunit (Supplementary Fig. 10).

#### Transfection of expression vectors and immunocytochemistry

We obtained CHO cells from the RIKEN Cell Bank (Tsukuba, Japan) and transfected these cells using FuGENE 6 (Boehringer Mannheim, Mannheim, Germany), as previously described [14].

For immunohistochemistry, cells were cultured in eight-well chambered slides (0.7 cm<sup>2</sup>/well; Thermo Fisher Scientific, Waltham, MA, USA) for 24 h and transfected using plasmid DNA (8  $\mu$ g) and 20  $\mu$ l Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The cells were cultured for 24 h, fixed with 4% paraformaldehyde for 30 min, and washed thrice with 20 mM HEPES (pH 7.5) containing 100 mM sodium chloride (NaCl) for 5 min. The fixed cells were blocked with 10% fetal bovine serum (FBS) in HEPES containing 0.4% Triton X-100 for 1 h. Immunocytochemistry was performed using a guinea pig antibody against rat gonadotropin subunit  $\alpha$  (1:4,000 dilution) and rabbit-IgGs against rat LH $\beta$  (1:2,000 dilution) and FSH $\beta$  (1:4,000 dilution), which were a kind gift from the National Institute of Diabetes and Digestive and Kidney Disease by Dr AF Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Primary antibodies were detected following incubation with secondary antibodies (AffiniPure, Cy-3-conjugated donkey anti-rabbit or Cy-5-conjugated donkey anti-guinea pig antibodies, 1:500 dilution for each, Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA). Immunofluorescence was observed using a BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan) or the FLUOVIEW FV1000 confocal microscope (Olympus, Tokyo, Japan).

#### Supply of expression vectors

All expression vectors encoding rodent FSH and LH can be obtained from the RIKEN BioResource Center (BRC: <http://dna.brc.riken.jp/en/furnish.html>) or the Bioresource Engineering Division ([http://www.riken.jp/en/research/labs/brc/bioresour\\_eng/](http://www.riken.jp/en/research/labs/brc/bioresour_eng/)). RDB numbers are RDB No. 15146 (pCX-puro-ratLH), RDB No. 15147 (pCX-puro-ratFSH), RDB No. 15148 (pCX-puro-mouLH), RDB No. 15149 (pCX-puro-mouFSH), RDB No. 15150 (pCX-puro-masLH), and RDB No. 15151 (pCX-puro-masFSH).

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