—Original—

Development of a recombinant murine luteinizing hormone-binding protein as a selective hormone inhibitor

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Abstract: Physiological levels of gonadotropins promote follicular development and ovulation. However, high luteinizing hormone (LH) levels associated with diminished ovarian reserve (DOR) and premature ovarian insufficiency (POI) can inhibit these processes. Here, we developed a mouse LH-binding protein (mLBP) as a selective inhibitor of mouse LH to ultimately permit *in vivo* analysis of the association between a high LH environment and follicular development disorders in mice with DOR or POI. mLBP was produced as a soluble protein by incorporating the extracellular-encoding domain of mouse LH receptor genes into an expression system for membrane-immobilized fusion proteins. The binding affinity of mLBP for mouse LH was confirmed using sera containing high levels of LH and follicle stimulating hormone (FSH) collected from ovariectomized (OVX) mice. The activity of mLBP was demonstrated by inhibition of cAMP and testosterone production induced by OVX-mouse serum in mouse Leydig-derived MLTC-1 cells expressing LH receptors. In contrast, mLBP neither bound mouse FSH nor inhibited cAMP production induced by OVX mouse serum in 293 cells expressing mouse FSH receptors. mLBP also exhibited binding affinity for human LH (hLH) and inhibited hLH-induced cAMP production in MLTC-1 cells. Thus, mLBP was demonstrated to selectively suppress the actions of mouse and human LH.

Key words: Luteinizing hormone, Luteinizing hormone receptor, Follicle stimulating hormone, Diminished ovarian reserve, Premature ovarian insufficiency

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Introduction

Ovarian reserve is defined as the number of residual oocytes in the ovary [1]. As both the quantity and quality of oocytes decline with age, the risk of infertility increases in aging females [2]. Diminished ovarian reserve (DOR) is a natural physiological symptom noted in most women in their early forties or earlier in cases of ovarian insufficiency [3, 4]. Premature ovarian insufficiency (POI) refers to a significantly diminished or absent ovarian reserve in 1–2% of women before the age of 40 years due to genetic, immunological, iatrogenic, or other causes. DOR and POI are characterized by impaired follicle development, resulting in ovulation disorders and subsequently oligomenorrhea and amenorrhea [5]. Most patients with infertility are resistant to conventional ovarian stimulation to promote follicle development [6]. Although increasing doses and/or dosing frequencies of gonadotropin preparations are often prescribed for infertile patients with DOR or POI [7], the efficacy of this approach is limited and it has high treatment costs [8].

A previous study proposed a luteinizing hormone (LH) threshold and ceiling concept in which abnormally elevated serum LH levels could lead to deterioration effects, including growth arrest of granulosa cells, induction of follicular atresia, premature follicular luteinization, and impaired ovarian follicle development [9]. In DOR and POI, the amount of estradiol produced from granulosa cells in ovarian follicles decreases because of fewer growing follicles, and reduced estradiol production decreases the negative feedback of estradiol at the pituitary gland and hypothalamus resulting in hypergonadotropinemia (high circulating follicle stimulating hormone [FSH] and LH levels) [10]. A recent study reported that patients with severe POI who developed hypergonadotropinemia

did not display follicular growth when treated with human menopausal gonadotropin (HMG). However, after the extremely elevated endogenous gonadotropin levels had been reduced using estrogen and progesterone treatments, simultaneous daily administration of purified urinary HMG or recombinant FSH and a gonadotropin-releasing hormone (GnRH) agonist, to maintain high serum FSH abundance and low LH levels over the long term, resulted in follicular growth and yielded oocytes under an *in vitro* fertilization-embryo transfer (IVF-ET) program [11]. Furthermore, an earlier *in vitro* study demonstrated that high LH levels impair FSH-dependent rat antral follicle growth, by suppressing the expression of FSH receptors in granulosa cells, and reduce the production of GDF-9, an oocyte-derived factor known to play important roles in follicular development [12]. These studies indicate that a high LH environment might be responsible for impaired follicle development in DOR and POI. However, both LH and FSH levels are high in DOR and POI patients; therefore, the association between a high LH environment and impaired follicle development is difficult to assess without the use of a selective LH inhibitor.

Although a selective human LH inhibitor has been reported, no such selective inhibitor for animal studies involving mice is presently available. To study the effects of high LH on follicle growth in animal models of DOR and POI, we sought to develop a mouse LH-binding protein (mLBP), an extracellular domain protein of the LH receptor [13] designed to exhibit selective inhibition of LH action in mice, which will ultimately permit *in vivo* analysis of the association between a high LH environment and follicular development disorders. The anti-LH activity of mLBP was evaluated using mouse Leydig-derived MLTC-1 cells that express LH receptors endogenously [14]. Moreover, the selectivity of mLBP for mouse LH was confirmed using a murine FSH receptor stably expressed in 293 cells. The binding ability and anti-LH activity of mLBP for human LH were also assessed.

Materials and methods

Expression vector construction for chimeric mouse LtCD8 receptor

Total RNA was extracted from C57BL/6J female mouse ovaries using an RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript III First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) (Thermo Fisher Scientific, Waltham, MA, USA) to synthesize cDNA from total RNA. KOD-Plus-Neo (Toyobo, Osaka, Japan) was used to amplify the extracellular domain (1-1,077 bp) of the coding region of the mouse *Lhcgr* gene (NCBI mRNA RefSeq: NM_013582.3) by PCR. PCR mixtures (50 µl) containing 100 ng cDNA, 200 mol/l dNTP, 1.5 mmol/l MgSO4, 1 × KOD plus Neo PCR buffer, 1 U KOD –plus-Neo, and respective primers (100 nmol/l each) were prepared. PCR was performed under the following conditions: denaturation, 25 cycles at 94°C for 2 min followed by 98°C for 10 s, 62°C for 30 s, and 68°C for 40 s. The forward primer (5'-ATGAATTC-CATCACCATCACCATCACATGGGGCGGCGGGTCCC-GGCTCTG-3') was designed with EcoR I recognition sequences, and His tag sequences were added to the 5' part of the coding region of the mouse *Lhcgr* gene, whereas the reverse primer (5'-TATGTCGACATCTTCA-CAGGGATTGAAAGCATCTG-3') was designed with a SalI recognition sequence added to the 5' region of interest. The extracellular domain of the mouse *Lhcgr* gene was subcloned into the pcDNA3.1(+)-human *LtCD8* plasmid [12], which was kindly provided by Professor Aaron J.W. Hsueh of Stanford University School of Medicine. LtCD8 is an artificial fusion protein bearing an extracellular domain of the human LH receptor (amino acids 1–358) flanked by a stretch of the thrombin receptor sequence (the thrombin cleavage site, amino acids 36–66 of the thrombin receptor, and the transmembrane and cytoplasmic regions of CD8 [amino acids 162 to the C terminus]).

The PCR products were linearized with EcoRI (Takara Bio Inc., Shiga, Japan) and SalI (Takara Bio Inc.), whereas the pcDNA3.1(+)-human *LtCD8* plasmid was linearized with EcoRI and XhoI (Takara Bio Inc.). Ligation high Ver.2 (Toyobo) was used to ligate the PCR products and transform them into *Escherichia coli* MAX Efficiency DH5α competent cells (Thermo Fisher Scientific) for cloning. The colonies were extensively purified into pcDNA3.1(+)-mouse *LtCD8* plasmids using the PowerPrep HP Plasmid Prep System (Origene, Rockville, MD, USA). The sequence of the inserted gene in the plasmid vector was confirmed by direct DNA sequencing using an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Cells

Human fetal kidney-derived Expi293F cells (Thermo Fisher Scientific), which were used to generate mLBP, were subjected to shaking at 125 rpm at 37°C under 8% CO_2 using Expi293F Expression Medium (Thermo Fisher Scientific).

For the functional assessment of mLBP, mouse Leydig-derived MLTC-1 cells (American Type Culture Collection) expressing LH receptors were used. The MLTC-1

cells were cultured at 37°C under 5% CO_2 in RPMI1640 (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Gibco BRL, Thermo Fisher Scientific), 100 units/ml penicillin, and 100 µg/ml streptomycin (Nacalai Tesque).

To confirm the selectivity of mLBP for gonadotropins, we established human 293 cells stably expressing mouse FSH receptors. The expression of human FSH and LH receptors in human 293 cells was detected by RT-PCR. Total RNA was reverse-transcribed using a PrimeScript RT reagent kit (Takara Bio Inc.) according to the manufacturer's instructions. PCR amplification was performed using a TB Green Premix Ex Taq II (Takara Bio Inc.) on a Thermal Cycler Dice Real Time System III (Takara Bio Inc.) according to the following protocol: 30 s at 95°C, followed by 40 cycles of 5 s at 30°C and 60 s at 60°C. The PCR primer sequences used in this study are listed in Table 1. Amplified products were confirmed by 3% agarose gel electrophoresis (Takara Bio Inc.). Following electrophoresis, the gels were stained with ethidium bromide and visualized under a UV (245 nm) transilluminator. After confirming the absence of both human FSH and LH receptors in native human 293 cells, we generated human 293 cells stably expressing mouse FSH receptors. pcDNA3.1(+) plasmid vector (Thermo Fisher Scientific) was enzymatically treated with BamHI (Takara Bio Inc.) and EcoRI and linearized. The coding region (NCBI mRNA RefSeq: NM_013523) of the pCMV6-mFsh receptor plasmid vector (Origene) was amplified by PCR using KOD-Plus-neo. The PCR products were mixed with the linearized pcDNA3.1(+) plasmid vector, and an infusion reaction was performed using an In-Fusion HD Cloning Kit (Clonetech Laboratories, Inc., Palo Alto, CA, USA). The reactants were transformed into competent *E. coli* DH5α cells. After cloning, the pcDNA3.1(+)-mFshr plasmid vector was purified. For transfection, 293 cells were seeded at a density of 1 × 10^6 cells/dish in a 60-mm dish and cultured in RPMI medium containing 10% FBS (complete medium) for one day. Thereafter, 2.5 µg/dish pcDNA3.1(+)-mFshr plasmid vector was transfected into the cells using GeneJuice Transfection Reagent (Merck, MA, USA). The next day, the cells were reseeded at 2 × 10^5 cells/90-mm dish and cultured for one day. The culture medium was changed to complete medium containing 500 µg/ml G-418 (Nacalai Tesque) for the application of selective pressure to the cells, and the medium was refreshed every 3–4 days to produce 293 cells stably expressing mouse FSH receptors.

Table 1. PCR primer sequences

Primer	Primer sequence (5' - 3')
Human FSHR forward	CATCATCGGATCTGTCACTGCTCTA
Human FSHR reverse	CTCGAAGCTTGGTGAGGACAAAC
Human LHR forward	CATTCAATGGGACGACACTGAC
Human LHR reverse	GGCCTGCAATTTGGTGGAAG
Mouse FSHR forward	CCAAGATAGCAAGGTGACCGAGA
Mouse FSHR reverse	CATGCAAGTTGGGTAGGTTGGA

Gene transfection and protein expression

pcDNA3.1(+)-human *LtCD8* and pcDNA3.1(+)-mouse *LtCD8* plasmid vectors were transfected into Expi293F cells using an ExpiFectamine 293 Transfection kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the ExpiFectamin 293 Reagent and Opti-Plex Complexation Buffer (Thermo Fisher Scientific) included in the kit were mixed and incubated at 25°C for 5 min. After adding each plasmid vector, the mixtures were allowed to react at room temperature for 10–20 min. Then, Expi293F cells were added to the mixtures and cultured in shaker flasks under the standard procedure of 125 rpm at 37°C under 8% CO_2. At 16–18 h after culture, enhancer reagents in the ExpiFectamine 293 Transfection kit were added, and the mixtures were cultured for one or two days.

Expi293F cells that were induced to express recombinant proteins were harvested and washed once with calcium- and magnesium-free phosphate-buffered saline. Subsequently, the cells were suspended in RPMI1640 medium containing 5 U/ml thrombin (Cytiva, Marlborough, MA, USA) and allowed to react in an incubator at 37°C for 1 h. Argatroban monohydrate (10 µmol/l; Tokyo Kasei Industry Co., Ltd., Tokyo, Japan) was added to arrest the cleavage response by thrombin. After centrifugation at 2,450 × g at 4°C, the culture supernatants were collected and ultrafiltered by centrifugation at 2,450 × g at 4°C using a Vivaspin 10-20K (Sartorius, Gottingen, Germany). Then, the supernatants were further concentrated using Amicon Ultra 0.5-10 K units (Merck) by centrifugation at 2,450 × g and 4°C to enrich the mLBP culture supernatant > 100-fold. The concentration of mLBP in the mLBP-enriched culture supernatant was measured using a DYKDDDDK-Tag Detection ELISA Kit (Cayman Chemicals, MI, USA), and the results were expressed as Flag-tagged mLBP concentrations. The mLBP-enriched culture supernatant was used for the mLBP treatment of each experimental group. The concentration of total protein in the mLBP-enriched culture supernatant was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The mLBP-enriched culture supernatants were stored at –80°C until use. Because the mLBP-

enriched culture supernatants contained thrombins and nonspecific proteins, the controls for the mLBP-enriched culture supernatant were prepared as a vehicle-enriched culture supernatant. After the pcDNA3.1(+) plasmid vector was introduced into Expi293F cells, the vehicle-enriched culture supernatant was prepared in the same manner as the mLBP-enriched culture supernatant. In each experiment, the vehicle-enriched culture supernatant was added to the treatment solution for the control groups to ensure that the total protein concentration in the treatment solution of the control group and that of the mLBP group were the same.

Immunoblot

Expi293F cells overexpressing recombinant proteins or supernatants after thrombin reaction were solubilized by adding Laemmli sample buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The proteins were SDS-equilibrated and resolved by SDS-PAGE using 4–15% (Fig. 1B) or 10% gels (Figs. 2B and 5B) for separation. Subsequently, they were transferred to a polyvinylidene difluoride membrane (Merck) at 200 V for 30 min. After blocking with 5% skim milk in PBS containing 0.05% Tween20 for 60 min, an anti-FLAG M2 mouse monoclonal antibody (dilution 1:1,000, Merck) was used as the primary antibody, and anti-mouse IgG HRP-conjugated antibody (dilution 1:10,000, Cell Signaling Technology, Beverly, MA, USA) was used as a secondary antibody. The ECL Prime western blotting Detection Reagent (Cytiva, Marlborough, MA, USA) was used to detect mLBP in the samples.

Animal experiments

Female ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All animals were housed in a room with controlled temperature (20–25°C), humidity (50–60%), and lighting (12-h light/dark cycle), with free access to a standard laboratory chow diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and tap water. All experimental procedures and animal housing conditions were approved by the Animal Care and Use Committee of the International University of Health and Welfare (approval number: 18022NA). Animal care was performed according to the guidelines of our institution and the National Institutes of Health.

To obtain ovariectomized (OVX) mice, we shaved both flanks of mice under isoflurane (Pfizer Japan Inc., Tokyo, Japan) inhalation anesthesia, dissected the skin of the right and left ovaries in a recumbent position to approximately 5 mm, and dissected the dorsal muscles beneath them. Both ovaries and fallopian tubes were exposed externally along with surrounding fat from the incisional orifice, ligated, and dissected between the fallopian tubes and uterine corpus using a nylon thread. Control animals underwent a sham operation for wound closure only after dissection of the skin and back muscles. Five weeks after the procedure, blood was sampled from the hearts of all animals under isoflurane anesthesia, and serum was recovered from the blood using a vacuum blood collection tube (Terumo Corporation, Tokyo, Japan).

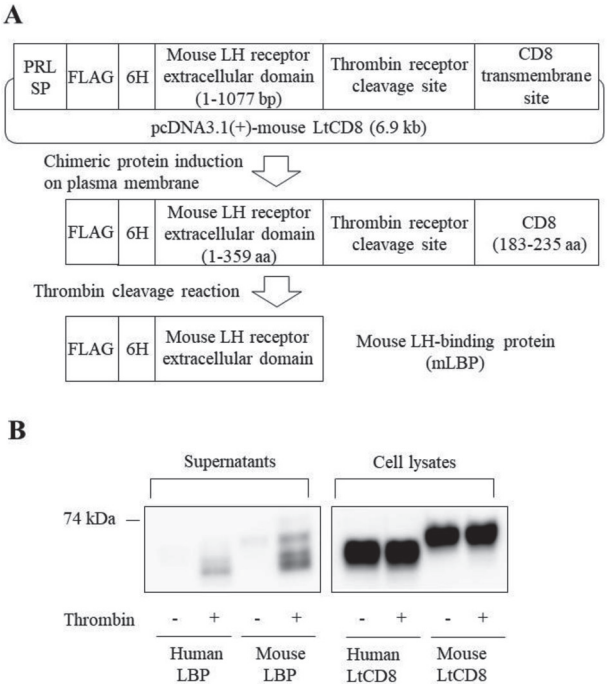

Fig. 1. Genetic construction and production of mouse LH-binding protein (mLBP).
A) Plasmid map of the mouse *LtCD8* fusion gene. bp: base pair, aa: amino acids, PRL SP: prolactin signal peptide, 6H: 6 × His.
B) Expression of LBPs and LtCD8 in Expi293F cells. Left: human and mouse LBPs released in cell culture supernatants after thrombin cleavage reaction. Right: human LtCD8 and mouse LtCD8 in cell lysates after thrombin cleavage reaction. Expi293F cells were transfected with pcDNA 3.1(+)-human *LtCD8* gene or pcDNA 3.1(+)-mouse *LtCD8* gene for 3 days. LBPs and LtCD8 were detected by immunoblotting using anti-FLAG antibody. Groupings of blots cropped from two parts of the same gel are displayed.

Hormonal assays

Mouse LH and FSH levels were measured using a Milliplex Mouse Pituitary Magnetic Bead Panel (Merck) according to the manufacturer's instructions. The Bio-

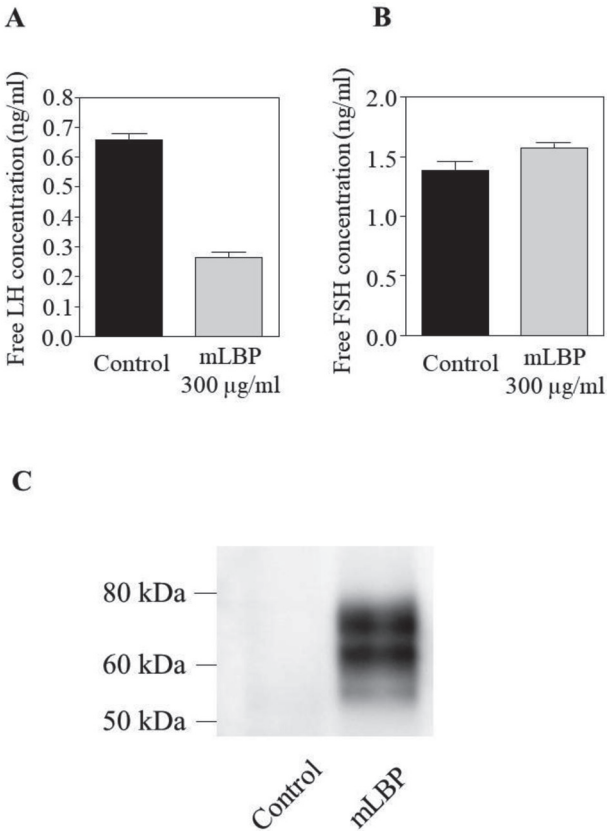

Fig. 2. Binding affinity of mLBP for mouse LH and FSH.
A) Free LH and FSH concentrations in the filtrate. In the control group, sera collected from ovariectomized (OVX) mice (12.5% of final concentration) and vehicle-enriched supernatant were mixed, whereas in the mLBP group, OVX-mouse sera (12.5% of final concentration) and mLBP-enriched culture supernatant (300 μg/ml of final concentration of Flag-tagged mLBP) were mixed. Each mixture was added to the anti-FLAG- M2 antibody-affinity gel to trap the complexes of mLBP and gonadotropins on the affinity gel, and the concentrations of unbound free gonadotropins in the filtrate were measured using the Milliplex Mouse Pituitary kit. Mean ± SD (n = 3 each).
B) Detection of mLBP eluted from anti-FLAG M2 antibody-affinity gel. In the control and mLBP groups, mLBP bound to anti-FLAG M2 antibody-affinity gel was eluted by competition using 3 × FLAG® peptide and detected by immunoblotting using an anti-FLAG antibody.

Plex 200 System (Bio-Rad Laboratories Inc.) and Bio-Plex Manager Software v.6.1 (Bio-Rad Laboratories Inc.) were employed as the measuring device and analysis software, respectively. The intra- and inter-assay variances of mouse LH and FSH were < 15% and 20%, respectively. The average minimum detectable concentrations of mouse LH and FSH were 4.9 and 24.4 pg/ml, respectively.

Human LH levels were measured using a Luteinizing Hormone Human ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. An EnVision Multilabel Reader (Perkin Elmer, Hopkinton, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) were used as the measuring device and analysis software, respectively. The intra- and inter-assay variances of human LH were less than 10% and 12%, respectively, and the average minimum detectable concentration was 156.3 pg/ml.

mLBP-ligand binding assay

To investigate the interaction of mouse gonadotropins and mLBP, an mLBP-ligand binding assay was performed. Because native LH purified from mice is not commercially available, the binding affinity of mLBP for mouse LH was confirmed using sera containing high levels of LH and FSH obtained from OVX mice (OVX-mouse serum). In the control group, 25 μl of OVX-mouse serum was incubated with 175 μl of vehicle-enriched supernatant (12.5% of the final concentration of OVX-mouse serum), whereas 25 μl of OVX-mouse serum was incubated with 175 μl of mLBP-enriched culture supernatant (343 μg/ml Flag-tagged mLBP) in the mLBP group. Similarly, to investigate interactions between human LH and mLBP, 100 μl of 2 ng/ml recombinant human LH (rhLH, R&D systems, Minneapolis, MN, USA) solution was incubated with 100 μl of vehicle-enriched supernatant in the control group, whereas 100 μl of 2 ng/mL rhLH solution was incubated with 100 μl of mLBP-enriched culture supernatant (200 μg/ml Flag-tagged mLBP) in the mLBP group. After 4 h of incubation at 4°C, each mixture was added to a column filled with anti-FLAG M2-antibody affinity gel (Merck). The filtrate was collected by centrifugation at 200 × g for 30 s. The levels of mouse LH and FSH, and human LH in the filtrates were determined as described above. The concentration of free gonadotropin in the filtrates decreased if the complexes of mLBP and gonadotropin were captured with an anti-FLAG M2-antibody affinity gel through the FLAG tag involved in mLBP. Then, the mLBP bound to the gel was competitively eluted with 150 μg/ml 3 × FLAG® peptide (Merck), and detected by immunoblotting using an anti-FLAG M2 antibody, as described above.

Measurement of cAMP levels

To evaluate the anti-mouse LH and FSH activities of mLBP, MLTC-1 and 293 cells stably expressing mouse FSH receptors (mFSHR) were used. These cells were seeded at a density of 1×10^4 cells/5 μl/well in each well

of a white round-bottomed 384-well plate (Greiner Bio-One, Frickenhausen, Germany) and treated with 5 µl of sham-operated or OVX-mouse sera (2.5% and 5% of final concentrations) for 1 h and then subjected to measurement of intracellular cAMP levels to confirm the activities of serum gonadotropins.

To determine the anti-mouse LH and FSH activities of mLBP, cells were treated with 5 µl of a mixture of OVX-mouse serum (5% of the final concentration) and different concentrations of mLBP-enriched culture supernatant (3, 10, 30, or 100 µg/ml of a final concentration of Flag-tagged mLBP). In the control group, cells were treated with 5 µl of a mixture of OVX-mouse serum (5% of the final concentration) and the vehicle-enriched culture supernatant (corresponding concentrations of total protein to each mLBP group) as controls. The anti-human LH activity of mLBP was also assessed using a similar approach.

Intracellular cAMP levels were measured using a homogeneous time-resolved fluorescence (HTRF®) assay kit (Perkin Elmer) according to the manufacturer's instructions. An EnVision Multilabel Reader and GraphPad Prism 9 were used to determine cAMP levels. For negative controls, cells were incubated with 1 × stimulation buffer included in the kit. The cAMP concentration of each sample was calculated by subtracting the mean cAMP concentration of the negative control group as the background. Subsequently, the relative % of the mean cAMP level in the treatment group relative to the mean cAMP level in the control group (control = 100%) was calculated, and the results are presented as the means ± SD (n = 4 each).

Measurement of testosterone levels

MLTC-1 cells were cultured in RPMI1640 medium with 10% steroid hormone-free FBS (DCC-FBS) prepared by dextran-coated charcoal treatment for 3 days, and then plated in 96-well plates at 5 × 10^4 cells/well. After 24 h of pre-culture, the cells were treated with RPMI1640 alone as a negative control group, 100 µl of mixtures of OVX-mouse serum (5% of the final concentration) and vehicle-enriched culture supernatant as the control group, OVX-mouse serum (5% of the final concentration) and 100 nmol/l abiraterone (a CYP17A1 inhibitor; Tokyo Kasei Industry Co., Ltd.) as a positive control group, and OVX-mouse serum (5% of the final concentration) and different concentrations of mLBP-enriched culture supernatant (10, 30, 100, or 300 µg/ml of a final concentration of Flag-tagged mLBP). The treatment medium contained 2 µmol/l pregnenolone (Tokyo Kasei Industry Co., Ltd.) as a testosterone synthesis substrate.

After one day of culture, testosterone levels in the cell culture supernatants were measured using a Testosterone HTRF® kit (Perkin Elmer) according to the manufacturer's instructions. An EnVision Multilabel Reader and GraphPad Prism 9 were used to measure testosterone levels. The mean testosterone concentration of the negative control group was subtracted from the testosterone concentration of each sample group. The relative % of the mean testosterone level in the treatment group relative to the mean testosterone level in the control group (control = 100%) was calculated, and the results are presented as the means ± SD (n = 4 each).

Cell viability

Cell viability was measured using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. MLTC-1 cells were cultured in RPMI1640 medium with 10% DCC-FBS for 2 days and then plated in 96-well plates at 5 × 10^4 cells/well. After 24 h of treatment of the MLTC-1 cells with OVX-mouse serum (5% of the final concentration) (negative control), or OVX-mouse serum (5% of final concentration) and cisplatin (300 µmol/l) (Tokyo Kasei Co., Ltd.) (positive control), or OVX-mouse serum (5% of the final concentration), and mLBP (300 µg/ml Flag-tagged mLBP), the cell numbers were counted after trypsin treatment (Nacalai Tesque) using a TC20 automatic cell counter (Bio-Rad Laboratories Inc.). To determine the cell viability, WST-8 reagent (10 µl) was added to the cell culture supernatants which were then incubated for 2 h, and the optical absorbance at 450 nm was measured using an EnVision Multilabel Reader (n = 4 each). The cell number and cell viability ratios of the experimental groups were compared with the mean measurement values of the control group, which served as a reference (% of control). To evaluate morphological changes, cellular images of each group were captured using a phase-contrast microscope (Nikon, Tokyo, Japan) when cells in the control group had proliferated to sub-confluency.

Statistical analysis

Data are presented as the mean ± SD. Comparisons of groups were performed using the Mann-Whitney U-test between two groups, or Bonferroni's multiple comparison test among multiple groups, with statistical significance being recognized as $P < 0.05$.

Results

Genetic construction and production of mLBP

To generate mLBP, an extracellular domain of the

mouse LH receptor, we modified the human *LtCD8* gene, a previously produced fusion gene [13]. The human *LtCD8* gene is designed to express the extracellular domain of the human LH receptor binding domain in the CD8 transmembrane domain through a thrombin cleavage site [13]. We changed the extracellular domain of the human LH receptor to the DNA sequence of the extracellular domain of the mouse LH receptor to construct the pcDNA3.1(+)-mouse *LtCD8* gene (Fig. 1A).

Expi293F cells transiently transfected with the pcDNA3.1(+)-human *LtCD8* gene and pcDNA3.1(+)-mouse *LtCD8* gene were treated with thrombin to free the soluble extracellular domains of human and mouse LH receptors in the cell culture supernatants. Soluble human and mouse LBPs (hLBP and mLBP, respectively) were detected by immunoblotting using an anti-FLAG antibody in the culture supernatant with thrombin, but were not be detected in the culture supernatant without thrombin (Fig. 1B). Human and mouse LtCD8 full-length proteins were detected at similar levels in cell lysates with or without thrombin treatment due to the presence of an excess amount of LtCD8 protein for thrombin cleavage (Fig. 1B).

Interaction between mLBP and mouse gonadotropins

After incubation with a mixture of OVX-mouse serum (12.5% of the final concentration) and mLBP-enriched culture supernatant (300 µg/ml Flag-tagged mLBP), which were added to the FLAG M2 antibody-affinity gel, the gonadotropin levels of the filtrates were measured. The free LH levels in the control and mLBP groups were 0.659 ± 0.019 and 0.265 ± 0.018 ng/ml, respectively, indicating a 59.8% reduction in LH immunoactivity following mLBP treatment (Fig. 2A). In contrast, no reduction in the free FSH level was found in the mLBP group (control: 1.382 ± 0.076 ng/ml vs. mLBP: 1.572 ± 0.040 ng/ml) (Fig. 2B). mLBP bound to anti-FLAG M2 antibody-affinity gel was subsequently eluted by competition using 3 × FLAG® peptide, and mLBP in the eluate was detected by immunoblotting using an anti-FLAG antibody (approximately 60 kDa) to confirm the binding of Flag-tagged mLBP to the gel (Fig. 2C). We attempted to measure the LH level in the eluate using a Milliplex Mouse Pituitary kit; however, it was below the detection limit of the kit (data not shown).

Cell bioassay to evaluate mLBP anti-LH activity

Using OVX-mouse serum and mouse MLTC-1 cells expressing LH receptors, we evaluated the anti-mouse LH activity of mLBP. We first confirmed the elevation of both LH and FSH levels in OVX-mouse serum. The LH and FSH levels in serum obtained from sham-operated mice (sham-mouse serum) were 0.32 ± 0.11 and 0.89 ± 0.50 ng/ml, respectively, and increased to 4.76 ± 1.52 and 26.21 ± 3.93 ng/ml in OVX-mouse serum, respectively. Notably, we confirmed that MLTC-1 cells do not induce cAMP production in response to recombinant mouse FSH (data not shown).

After confirming cAMP production was induced by the treatment of MLTC-1 cells with 10 µmol/l forskolin as a positive control (data not shown), we measured the intracellular cAMP levels of the different treatment groups. After 1-h culture of MLTC-1 cells in a medium containing OVX-mouse serum at 2.5% and 5% final concentrations, corresponding to 0.12 and 0.24 ng/ml of LH in the medium, respectively, and in a medium containing sham-mouse serum at 2.5% and 5% final concentrations, corresponding to 0.01 and 0.02 ng/ml of LH in the medium, respectively, intracellular cAMP production increased in a LH concentration-dependent manner (Fig. 3A). The mLBP-enriched culture supernatant inhibited cAMP production induced by OVX-mouse serum (5% of the final concentration) in a concentration-dependent manner (Fig. 3B). Although monitoring intracellular cAMP production is a sensitive and high-throughput method for evaluating the anti-LH activity of mLBP, cAMP production could have been affected by other factors derived from the mouse sera. Therefore, we also assessed testosterone production as a specific downstream factor of LH action in testicular Leydig cells. The testosterone concentration in the supernatants of MLTC-1 cells treated with a medium containing sham-mouse serum at a 5% final concentration was 39.4 ± 10.9 nmol/l and increased to 855.0 ± 234.4 nmol/l when the cells were treated with a medium containing OVX-mouse serum (5% of final concentration). In the positive control group, testosterone production induced by OVX mouse serum was almost completely inhibited by 100 nmol/l abiraterone, a small-molecule inhibitor of cytochrome P17 (CYP17), which serves as a key enzyme in testosterone production (Fig. 3C). mLBP-enriched culture supernatants inhibited testosterone production induced by OVX-mouse serum in a concentration-dependent manner (Fig. 3C). To clarify the potentially deleterious effects of mLBP on MLTC-1 cells, we confirmed the viability of MLTC-1 cells at the end of the culture period. Although the cell number and viability decreased in the cisplatin group as previously reported [14], the number of live cells and cell viability in the group treated with the mLBP-enriched culture supernatant at the maximum effective concentration (300 µg/ml Flag-tagged mLBP) were comparable to those of the control group (Figs. 3D and E). Furthermore, the cells

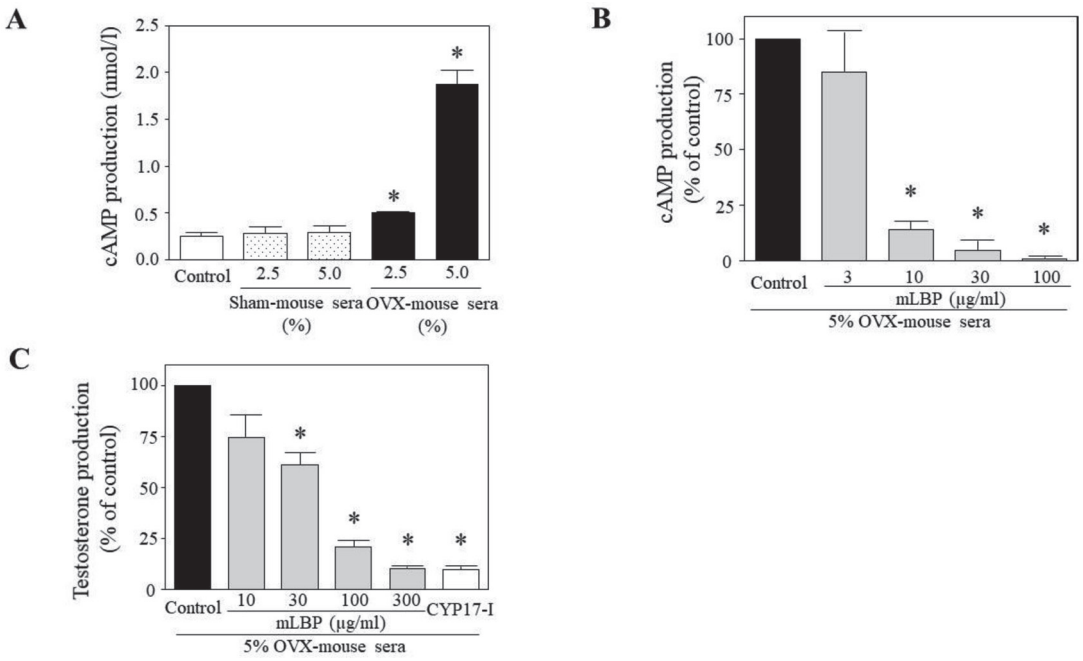

Fig. 3. Inhibitory effects of mLBP on cAMP and testosterone production induced by sera from ovariectomized mice in MLTC-1 cells. A) cAMP production in sera obtained from ovariectomized mice. MLTC-1 cells were treated with sera obtained from sham-operated or ovariectomized mice (sham-mouse or OVX-mouse sera) for 1 h, and intracellular cAMP concentrations were measured using a homogeneous time-resolved fluorescence (HTRF®) assay kit. In the control group, cells were incubated with an assay buffer. The relative % of the mean cAMP level in the treatment group relative to the mean cAMP level in the control group (control = 100%) is presented. Means ± SD (Bonferroni's multiple comparison test, $*P < 0.05$ vs. control, n = 4 each). B) Inhibitory effect of mLBP on cAMP production induced by OVX-mouse serum. Cells were treated with OVX-mouse serum (5% of final concentration) and mLBP-enriched culture supernatants (indicated by Flag-tagged mLBP) for 1 h, and intracellular cAMP levels were measured. The relative % of cAMP levels in the mLBP group to the level in the control group (control = 100%) is presented. Means ± SD (Bonferroni's multiple comparison test, $*P < 0.05$ vs. control, n = 4 each). C) Inhibitory effect of mLBP on testosterone production induced by OVX-mouse serum. Cells were treated with OVX-mouse sera (5% of final concentration) and mLBP-enriched culture supernatant (indicated by Flag-tagged mLBP) or 100 nmol/l CYP17A1 inhibitor, abiraterone (CYP17-I). After 24 h, testosterone levels in the supernatants were measured using a HTRF® assay kit. The relative % of the testosterone level in the mLBP or CYP17-I group to the control group (control = 100%) is presented. Means ± SD (Bonferroni's multiple comparison test, $*P < 0.05$ vs. control, n = 4 each).

proliferated to sub-confluency in the control and mLBP groups and showed no abnormal morphological changes in a comparison with the cisplatin group, which exhibited limited proliferation ability and apoptotic morphology (Fig. 3F).

Effects of mLBP on mouse FSH activity

Because there is no commercially available cell line that specifically responds to mouse FSH, we generated a human cell line stably expressing the mouse FSH receptor (Fig. 4A) to confirm the specificity of mLBP. We confirmed that human 293 cells expressed neither human FSH nor LH receptors (Fig. 4A), and did not induce cAMP production in response to recombinant human LH stimulation (data not shown).

After 1-h of culture of 293 cells in a medium containing OVX-mouse serum at 2.5% and 5% final concentrations, corresponding to 0.66 and 1.31 ng/ml FSH in the medium, respectively, and in a medium containing sham-mouse sera at 2.5% and 5% final concentrations, corresponding to 0.02 and 0.04 ng/ml of FSH in the medium, respectively, intracellular cAMP production increased in an FSH concentration-dependent manner (Fig. 4B). However, treatment with mLBP-enriched culture supernatants (10, 30, and 100 μg/ml Flag-tagged mLBP) for 1 h did not perturb cAMP production in 293 cells cultured in a medium containing OVX-mouse serum (5% of final concentration) (Fig. 4C).

Anti-LH activity of mLBP on recombinant human LH

We also evaluated the binding affinity of mLBP for hLH. After incubation with a mixture of rhLH (1 ng/ml final con-

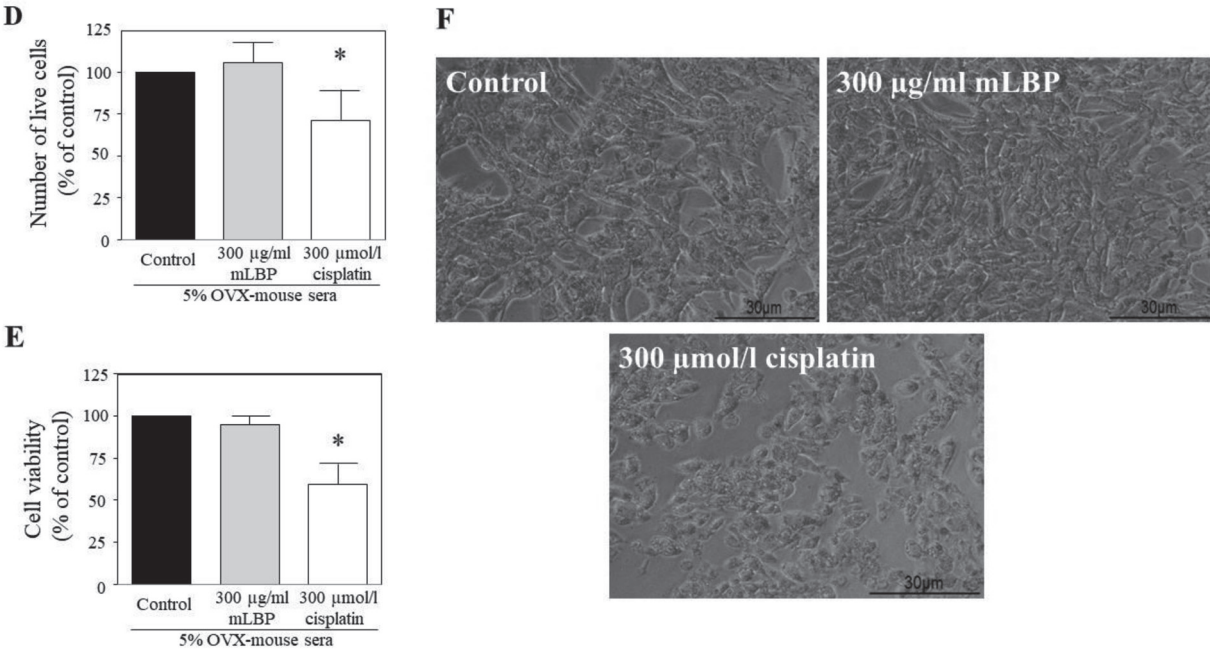

Fig. 3. (continued)
D-F) Effect of mLBP on cell viability. Cells were treated with OVX-mouse sera (5% of final concentration), mLBP-enriched culture supernatants (300 µg/ml of final concentration of Flag-tagged mLBP) or 100 µmol/l cisplatin for 24 h. The number of live cells (D) was counted using a cell counter, and the cell viability (E) was measured using a cell counting kit. The relative % of cell numbers or absorbance in each group to the control (control = 100%) is presented. Mean ± SD (Bonferroni's multiple comparison test, **P* < 0.05 vs. control, n = 4 each). Representative cell morphology in each group (F).

centration) and mLBP-enriched culture supernatant (100 µg/ml Flag-tagged mLBP) or vehicle-enriched culture supernatant (control), samples were added to the FLAG M2 antibody-affinity gel, and the free hLH levels of the filtrates were measured. The concentration of free hLH in the mLBP group was lower than that in the control cohort (0.195 ± 0.003 vs. 0.905 ± 0.005 ng/ml, respectively), indicating a 78.5% reduction in hLH immunoreactivity following mLBP treatment (Fig. 5A). mLBP bound to anti-FLAG M2 antibody-affinity gel was subsequently eluted by competition using 3 × FLAG® peptide, and mLBP in the eluate was detected by immunoblotting using an anti-FLAG antibody (approximately 60 kDa) to ensure the binding of Flag-tagged mLBP to the gel (Fig. 5B). However, the LH level in the eluate could not be measured by ELISA as it was below the detection limit (data not shown).

Next, we investigated whether mLBP could suppress LH activity in MLTC-1 cells. MLTC-1 cells have been reported to react with human LH to produce cAMP [15]. As reported previously, cells were cultured for 1 h in a medium containing 1 ng/ml rhLH and a significant increase in intracellular cAMP production (Fig. 5C) was observed. mLBP-enriched culture supernatants inhibited cAMP production induced by 1 ng/ml rhLH in a concentration-dependent manner (Fig. 5D).

Discussion

In this study, we generated mLBP, the ligand-binding region of the mouse LH receptor, by incorporating the extracellular domain of murine LH receptor into an expression system involving a previously developed membrane-immobilized fusion proteins [13]. mLBP acted as a novel agent that exhibited binding affinity for mouse LH and inhibited LH receptor signals activated by LH as a functional antagonist. mLBP showed no affinity for mouse FSH and acted as a soluble agent that selectively inhibited LH activity. Furthermore, the potential of direct deleterious effects of mLBP to attenuate LH receptor signals in MLTC-1 cells could be dismissed due to the absence of cellular toxicity of mLBP. Therefore, mLBP has the potential to serve as a powerful tool in the analysis of the roles of high LH levels in follicular development and ovulation in *in vivo* animal models of DOR and POI [16, 17].

Currently, two different types of agents modulate gonadotropin (FSH and LH) secretion. One is estrogen/pro-

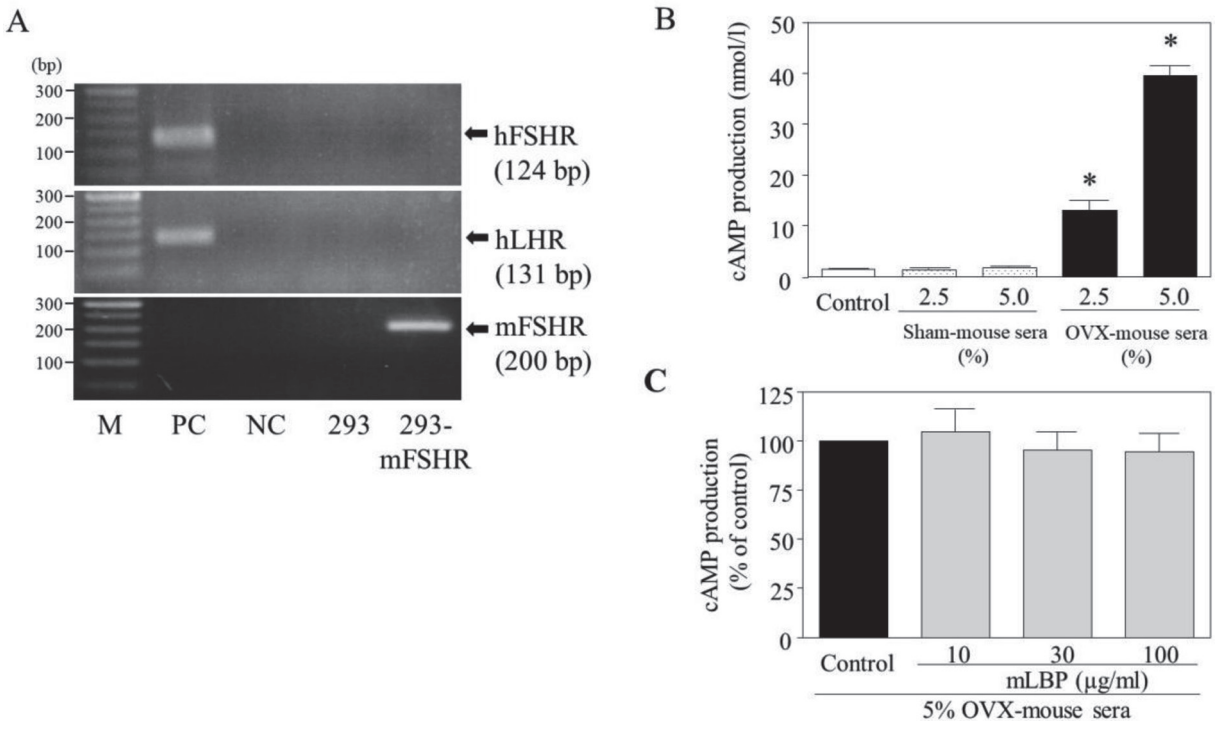

Fig. 4. Effects of mLBP on FSH activity in 293 cells stably expressing mouse FSH receptors.
A) Expression of FSH and LH receptors in 293 cells. The expression of FSH and LH receptors was detected by RT-PCR. hFSHR: human FSH receptor, hLHR: human LH receptor, mFSHR: mouse FSH receptor, M: DNA ladder, PC: positive control (human ovary cDNA), NC: negative control (total RNA of 293 cells without RT), 293: 293 cells, 293-mFSHR: 293 cells stably expressing mouse FSH receptors.
B) cAMP production in sera obtained from ovariectomized mice. The 293 cells stably expressing mouse FSH receptors were treated with sera from sham-operated or ovariectomized mice (sham-mouse or OVX-mouse sera) for 1 h, and intracellular cAMP levels were measured using a homogeneous time-resolved fluorescence (HTRF®) assay kit. Mean ± SD (Bonferroni's multiple comparison test, $*P < 0.05$, n = 4 each).
C) Effect of mLBP on cAMP production induced by OVX-mouse serum. Cells were treated with OVX-mouse sera (5% of final concentration) and mLBP-enriched culture supernatants (indicated by Flag-tagged mLBP) for 1 h, and intracellular cAMP levels were measured. The relative % of cAMP levels in the mLBP group to the level in the control group (control = 100%) is presented. Mean ± SD (Bonferroni's multiple comparison test, $*P < 0.05$, n = 4 each).

gesterone drugs that promote negative feedback to the hypothalamus and pituitary gland, and the other is GnRH agonists and antagonists that act on GnRH receptors in the pituitary gland. Although these drugs are effective in regulating gonadotropin secretion, they simultaneously change both FSH and LH production. Therefore, in the clinical market, there is no available drug that only targets LH action. In preclinical studies, the development of selective FSH inhibitors, including small-molecule compounds, peptides, and antibodies against the FSH-receptor, has been reported. A peptide that inhibits the binding of FSH to its receptor and an anti-FSH beta monoclonal antibody have been reported to suppress FSH action in mice [18, 19]. As a selective LH inhibitor, BAY-298, a small-molecule allosteric modulator of the LH receptor, has also been reported, but this inhibitor does not act on mouse LH receptors [20]. Therefore, the mLBP described here is the first selective mouse LH-specific inhibitor.

A previous study reported the development of a soluble LH-binding protein, human LBP (hLBP), using the human LH receptor extracellular domain [13]. However, unlike rat LH receptors (which are highly homologous to murine LH receptors) which recognize LH and chorionic gonadotropin of rats, humans, equine, and ovine species, the human LH receptor has been shown to only recognize human LH and chorionic gonadotropin [21]. Indeed, we confirmed that OVX-mouse serum (final concentration up to 20%) did not induce cAMP production in 293 cells stably expressing human LH receptors (data not shown). Therefore, hLBP might not be suitable for *in vivo* studies in mice because of its low binding affinity for rodent LH.

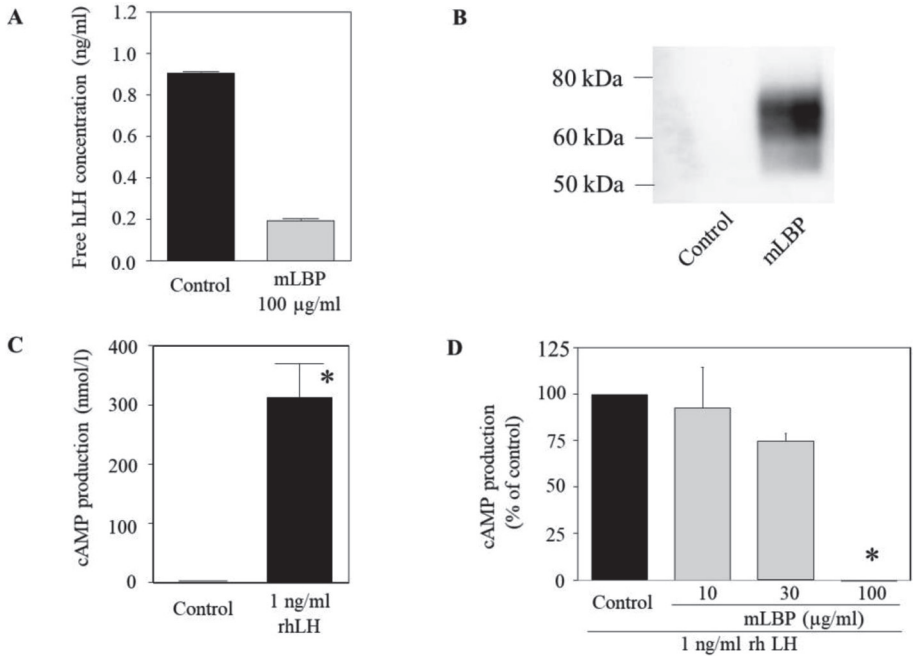

Fig. 5. Anti-LH activity of mLBP on recombinant human LH.
A) Binding affinity of mLBP for recombinant human LH (rhLH). In the control group, rhLH (1 ng/ml of final concentration) and vehicle-enriched supernatant were mixed, whereas in the mLBP group, rhLH (1 ng/ml of final concentration) and mLBP-enriched culture supernatant (100 µg/ml of final concentration of Flag-tagged mLBP) were mixed. Each mixture was added to the anti-FLAG M2 antibody-affinity gel to trap the complexes of rhLH and mLBP on the affinity gel, and the concentration of unbound free rhLH in the filtrate was measured using a human LH ELISA kit. Mean ± SD (n = 3 each).
B) Detection of mLBP eluted from the anti-FLAG M2 antibody-affinity gel. mLBP bound to anti-FLAG M2 affinity gel was eluted by competition using 3 × FLAG® peptide, and detected by immunoblotting with anti-FLAG antibody.
C) cAMP production by rhLH. MLTC-1 cells were treated with 1 ng/ml rhLH, or an assay buffer in the control group for 1 h. Intracellular cAMP levels were measured using a homogeneous time-resolved fluorescence (HTRF®) assay kit. Mean ± SD (Mann-Whitney U-test, $*P < 0.05$ vs. control, n = 4 each).
D) Inhibitory effect of mLBP on rhLH-induced cAMP production. MLTC-1 cells were treated with 1 ng/ml rhLH and mLBP-enriched culture supernatants (indicated by Flag-tagged mLBP) for 1 h, and intracellular cAMP levels were measured. The relative % of cAMP levels in the mLBP group to the level in each control group (control = 100%) is presented. Mean ± SD (Bonferroni's multiple comparison test, $*P < 0.05$ vs. control, n = 4 each).

As expected, based on the findings of the previous report, mLBP displayed binding affinity not only for mouse LH but also for human LH.

Although the earlier study did not prepare large quantities of hLBP, the baculovirus-insect cell system was used to obtain a large quantity of soluble human FSH-receptor extracellular domain expressed as a membrane-immobilized fusion protein [13]. In that system, post-translational modifications similar to those in mammalian systems could be added to the recombinant protein. However, preparation of the recombinant baculovirus takes a long time, and furthermore, there is a risk of residual baculovirus contamination in the purified recombinant protein. In the present study, we used Expi293F cells derived from human fetal kidney tissue as host cells for mouse LtCD8 expression. The Expi293F cell line shows high transfection efficiency and has been adapted for suspension growth of cells in serum-free medium [22]. Thus, mLBP can be prepared in large quantities in a short time frame by transient transfection. This system also has the advantage of allowing the direct administration of recombinant proteins to animals without the risk of viral contamination.

Testosterone production in primary cultures of mouse and rat testicular Leydig cells [23, 24] and aromatase activity in primary cultures of granulosa and Sertoli cells [21] are well-established bioassays that have been used to evaluate the activities of LH and FSH. However, these methods require the recovery of cells from sacrificed animals. Therefore, we developed simple and rapid bioassays to assess the activities of LH and FSH using MLTC-1 and 293 cell lines stably expressing mouse FSHR. By combining these cells, we established assays that could

selectively assess the bioactivity of LH in mouse serum and demonstrated the selective anti-LH activity of mLBP. mLBP inhibited testosterone production to an extent similar to that of the CYP19A1 inhibitor, abiraterone, a drug used in castration-resistant prostate cancer patients, indicating the high potential of mLBP as an LH inhibitory agent. Furthermore, we confirmed that mLBP showed no cytotoxicity, indicating that the inhibitory effect of mLBP was due to LH sequestration by mLBP and not a direct inhibitory effect of mLBP against these cells. mLBP also has a lower risk of toxic effects in *in vivo* studies.

To confirm the anti-LH activity of mLBP in *in vitro* studies, mLBP produced in cell culture supernatants after thrombin response was prepared on a small scale by concentrating the culture supernatant without purification, as described in a previous study [13]. Although we attempted to prepare purified mLBP using FLAG M2-affinity gel, the purified mLBP exhibited no anti-LH activity. This might have been the result of a decrease in the binding affinity of mLBP for ligands during the purification process, because the LH receptor has been reported to bind ligands via weak intermolecular interactions, such as ionic binding [25, 26]. Different mLBP purification methods will need to be evaluated in the future to establish the optimal method for purifying functional mLBP.

In mLBP-ligand binding assays, LH in OVX-mouse serum and rhLH were captured with mLBP on the gel, resulting in a decline in the free LH concentration in the filtrate. To confirm the binding of mLBP to the FLAG affinity gel, we assayed mLBP in the eluate by immunoblotting. Although we also attempted to determine LH levels in the eluate, the LH levels were below the assay detection limit. It is possible the anti-LH antibody might not have been able to detect LH due to the formation of complexes involving mouse and human LH with mLBP changing the antigen recognition sites.

In patients with DOR and POI, a high gonadotropin environment appears to be involved in the disorders of follicular growth and ovulation. However, experiments to selectively ameliorate high LH levels in mice with DOR and POI are necessary to elucidate the effects of a high LH level on impaired follicular growth and ovulation. In addition to DOR and POI, the inhibition of excessive LH levels may represent a valuable therapeutic strategy for other human pathologies, such as prostate cancer, polycystic ovarian syndrome, and precocious puberty.

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