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—Original Article—

Estrogen increases *KISS1* expression in newly generated immortalized *KISS1*-expressing cell line derived from goat preoptic area

Yukina OSHIMO[1)], Arisa MUNETOMO[1)], Fumie MAGATA[1)], Yuta SUETOMI[2)], Shuhei SONODA[3)], Yukari TAKEUCHI[3)], Hiroko TSUKAMURA[4)], Satoshi OHKURA[2)] and Fuko MATSUDA[1)]

[1)]*Laboratory of Theriogenology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*

[2)]*Laboratory of Animal Production Science, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*

[3)]*Laboratory of Veterinary Ethology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*

[4)]*Laboratory of Animal Reproduction, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*

Abstract. Kisspeptin neurons located in the hypothalamic preoptic area (POA) are suggested to be responsible for the induction of the gonadotropin-releasing hormone (GnRH) surge and the following luteinizing hormone (LH) surge to regulate female mammals' ovulation. Accumulating evidence demonstrates that the preovulatory level of estrogen activates the POA kisspeptin neurons (estrogen positive feedback), which in turn induces a GnRH/LH surge. This study aimed to derive a cell line from goat POA kisspeptin neurons as an *in vitro* model to analyze the estrogen positive feedback mechanism in ruminants. Neuron-derived cell clones obtained by the immortalization of POA tissue from a female Shiba goat fetus were analyzed for the expression of kisspeptin (*KISS1*) and estrogen receptor α (*ESR1*) genes using quantitative real-time reverse transcription-polymerase chain reaction and three cell clones were selected as POA kisspeptin neuron cell line candidates. One cell line (GP64) out of the three clones showed significant increase in the *KISS1* level by incubation with estradiol for 24 h, indicating that the GP64 cells mimic endogenous goat POA kisspeptin neurons. The GP64 cells showed immunoreactivities for kisspeptin and estrogen receptor α and retained a stable growth rate throughout three passages. Further, intracellular calcium levels in the GP64 cells were increased by the KCl challenge, indicating their neurosecretory ability. In conclusion, we generated a new *KISS1*-expressing cell line derived from goat POA. The current GP64 cell line could be a useful model to elucidate the estrogen positive feedback mechanism responsible for the GnRH/LH surge generation in ruminants.

Key words: Estrogen positive feedback, Kisspeptin, Ovulation, POA, Ruminant

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Reproductive functions in mammals are regulated by the hypothalamic-pituitary-gonadal axis: The gonadotropin-releasing hormone (GnRH) secreted from the hypothalamic GnRH neurons stimulates the pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion to enhance follicular development and steroidogenesis in the ovaries [1]. The high level of circulating estrogen secreted from mature follicles positively feedbacks to the hypothalamus to evoke surge mode secretion of GnRH and LH (GnRH/LH surge) to induce ovulation [2]. It has been reported that ovulatory dysfunctions in cattle are partly due to the insensitivity of the hypothalamus to the estradiol positive feedback [3]. Ovarian cyst, one of the major ovulation disorders, is estimated to occur in 10–13% of dairy cattle [4] and considered to be a major cause of cattle infertility. Further, it has been reported that approximately 10% of ovulation disorders in women are caused by hypothalamic pituitary failure, such as hypothalamic amenorrhea and hypogonadotropic hypogonadism [5]. A study conducted on anovulatory women with irregular menstruation (dysfunctional uterine bleeding) suggested that the ovulation failure is caused by decreased hypothalamic sensitivity to the estrogen positive feedback [6]. Thus, the molecular and cellular mechanisms underlying the estrogen positive feedback to regulate GnRH/LH surge should be elucidated in ruminants as well as women.

Several lines of evidence indicate that kisspeptin neurons play a key role to control reproduction via stimulating the GnRH release from GnRH neurons that express the kisspeptin receptor, GPR54 [7, 8] in mammals, including rodents, ruminants, and primates [9–14]. Indeed, *Kiss1* (encoding kisspeptin) knock out (KO) mice and rats never showed puberty onset, and the KO rats failed to show both LH pulses and estrogen-induced LH surge [15–18]. Further, GPR54 gene mutation caused infertility in human [19, 20]. In ruminants, such as goats, sheep, and cattle, and primates, kisspeptin neurons are located

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Correspondence: F Matsuda (e-mail: afukomat@g.ecc.u-tokyo.ac.jp)

in two hypothalamic regions: one is the preoptic area (POA) of the anterior hypothalamus and the other is the arcuate nucleus (ARC) in the mediobasal hypothalamus [21–25]. Kisspeptin expression in the ARC kisspeptin neurons, also called KNDy neurons that stand for the three neuropeptides expressed in these neurons (kisspeptin, neurokinin B, and dynorphin A), are negatively controlled by estrogen via estrogen receptor α (ERα) [24, 26, 27]. By contrast, kisspeptin neurons in the anterior hypothalamus, e.g., the POA in ruminants and primates or the anteroventral periventricular nucleus (AVPV) in rodents, expressing ERα, are positively regulated by estrogen [28–30]. It has been reported that nearly all the *Kiss1*-positive cells in the AVPV co-express ERα and estradiol treatment significantly increased the number of *Kiss1*- and kisspeptin-positive cells in the AVPV in mice and rats [28, 31]. The number of POA kisspeptin neurons co-expressing c-Fos, a marker for neuronal activation, increased at the time of LH surge in ewes [32], and the high 17β-estradiol (E_2) treatment increased the number of c-Fos-positive POA kisspeptin neurons in goats and monkeys [22, 33]. Thus, the POA/AVPV kisspeptin neurons are assumed to be a direct target of estrogen positive feedback and the central ovulation regulator [34, 35].

To examine the molecular and cellular neuroendocrine mechanisms mediating the central regulation of ovulation, *in vitro* experiment using neuronal cells derived from POA/AVPV kisspeptin neurons would be valuable, which enables us to directly analyze their characteristics and functions. In mice and rats, primary-cultured neuronal cells or brain slices can be used for *in vitro* kisspeptin neuron analysis [36–40]. Immortalized AVPV kisspeptin neuron cell lines derived from *Kiss1*-green fluorescent protein (GFP) transgenic mice have also been established and used as *in vitro* models [41, 42]. KTaV-3, a mouse AVPV kisspeptin neuron cell line, is confirmed to increase the *Kiss1* mRNA expression level to approximately 4–9 times by E_2 treatment [41], and considered to be a valuable tool to investigate the estrogen positive feedback mechanisms at cellular level. We have recently established the goat ARC kisspeptin neuron-derived immortalized cell line [43], but the ruminant-derived POA kisspeptin neuron cell lines have not been available yet. The ruminant-derived POA kisspeptin neuron cell lines are assumed to be quite useful because repeated primary cell culture of brains or preparation of brain slices is much more difficult in ruminants than in rodents.

The present study aimed to establish an immortalized cell line derived from goat POA kisspeptin neurons, which would be a useful tool to investigate estrogen positive feedback mechanisms in ruminants *in vitro*. Neuron-derived cell clones obtained by the immortalization of POA tissue from a female Shiba goat fetus [43] were analyzed for the expression of *KISS1* and estrogen receptor α gene (*ESR1*) mRNAs, as POA kisspeptin neuron markers, and screened according to their mRNA expression levels. Next, the effects of E_2 treatment on *KISS1* levels in the cell clones were evaluated to select the cell line(s) retaining the estrogen positive feedback system. The selected goat POA kisspeptin neuron cell line candidate was subjected to immunohistochemistry analysis for kisspeptin and ERα to evaluate their expression at the peptide/protein level. The growth rate of the selected goat POA kisspeptin neuron cell line candidate was also examined. Furthermore, the change in intracellular calcium levels after a KCl challenge in the cell line candidate was observed to show its ability to respond to depolarization.

Materials and Methods

Animals and tissue collection

The hypothalamus was procured from a female Shiba goat fetus to obtain immortalized cells as described in our previous report [43]. A goat fetus was used to obtain hypothalamic tissue since fetal tissues are generally used for culturing neuronal cells. This is because of their higher viability compared to those from adult tissues. Additionally, fetal hypothalamus has been used for the analysis of primary-cultured rodent kisspeptin neurons [36–40] as well as for the generation of mice kisspeptin neuron-derived immortalized cell lines [41, 42]. The fetal goat hypothalamus was divided into the rostral and caudal part at the optic chiasm. The rostral part including the POA was subjected to primary culture to obtain immortalized POA kisspeptin neurons after appropriate trimming. The rostral hypothalamic tissue was carefully prepared not to include the ARC region as much as possible. It is notable that the remaining caudal part of the fetal hypothalamus had been used to establish an immortalized ARC KNDy neuron cell line and one of the cell clones was reported as the KNDy neuron model (GA28) in our previous paper [43].

The ARC and POA tissues were taken from another goat to obtain cDNA for the positive control of reverse transcription-polymerase chain reaction (RT-PCR) analysis. A mature female Shiba goat was euthanized with 1.2 mg of xylazine (Ceractal; Bayer Yakuhin, Tokyo, Japan) and an overdose of sodium pentobarbital (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan). The POA and ARC regions were dissected and used for total RNA extraction and cDNA synthesis. This female Shiba goat was pregnant at the time of sampling, although the fetal age was unknown.

All experiments were performed according to the guidelines for the Care and Use of Laboratory Animals recommended by the Nagoya University and Japan. All experimental protocols and procedures were reviewed and approved by the Committee on the Care and Use of Experimental Animals of the Graduate School of Bioagricultural Sciences, Nagoya University.

Generation of neuron-derived cell clones and cell culture

Fetal goat POA-derived cell clones and their cDNA obtained in our previous study [43] were used. Briefly, primary-cultured POA tissue of a female Shiba goat fetus was immortalized using a lentivirus loaded with the simian virus 40 (SV40) large T-antigen gene and the neomycin resistance gene (GenTarget, San Diego, CA, USA). After selection by Geneticin (G418; Roche Diagnostics, Basel, Switzerland), the immortalized cell population was seeded onto poly-L-lysine-coated 96-well plates (AGC Techno Glass, Shizuoka, Japan) at a concentration of 1 cell/100 µl/well. The cell clones, which were confirmed to be derived from a single cell, were re-plated into poly-L-lysine-coated 24-well plates (AGC Techno Glass) and these cells were defined as passage number 0 (P0). The obtained 80 cell clones were named GP cells (GP1–GP88; eight cell clones were lost due to insufficient growth before the RNA extraction and/or preservation of freeze stock). The total RNA was extracted and cDNA was synthesized from the 80 cell clones, and subsequently, 48 cell clones were selected as neuron-derived cell clones by measuring the expression of neuron and glial marker genes (neuron-specific enolase gene and glial fibrillary acidic protein gene, respectively). The 48

neuron-derived cell clones were used to find the goat POA-derived kisspeptin neuron cell line in the present study.

The immortalized cell clones were plated on poly-L-lysine-coated plates (AGC Techno Glass) and cultured in the Neurobasal medium (Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan, Lot. 15N 353), B-27 Serum-Free Supplement (Thermo Fisher Scientific), 0.5 mM L-glutamine (Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific), and 5 ng/ml basic fibroblast growth factor (Thermo Fisher Scientific) at 37°C with 5% CO_2.

Total RNA extraction and cDNA synthesis from cell clones and goat POA and ARC tissues

From the P0 cell clones, the total RNA was extracted and cDNA was synthesized as described previously [43]. The cDNA was used for the screening by quantitative real-time RT-PCR (qRT-PCR) of *KISS1* and *ESR1*. From the cell clones after P1, total RNA was extracted using the ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized from 5 µg of total RNA treated with TURBO™ DNase (Thermo Fisher Scientific) using ReverTra Ace (TOYOBO, Osaka, Japan). The cDNA was then treated with RNaseH (Takara Bio, Shiga, Japan) to remove RNA and used for qRT-PCR to quantify the *KISS1* levels after E_2 treatment and for RT-PCR.

Total RNA was extracted from the goat POA and ARC tissues using the TriPure Isolation Reagent (Roche Diagnostics). Total RNA of the POA tissue was treated with TURBO™ DNase I, and cDNA was synthesized from 5 µg of the total RNA using ReverTra Ace, which was subsequently treated with RNaseH. Total RNA of the ARC tissue was treated with Amplification Grade DNase I (Thermo Fisher Scientific), and cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA). The cDNA was used as the positive control of RT-PCR analysis.

qRT-PCR for KISS1 and ESR1 to select goat POA kisspeptin neuron cell line candidates

The *KISS1* and *ESR1* expression was quantified by qRT-PCR. Actin beta gene (*ACTB*) was used as an internal reference. The qRT-PCR was performed in triplicate by a StepOnePlus Real-Time PCR system (Applied Biosystems) using the THUNDERBIRD probe qPCR Mix (TOYOBO). Sequences of primers and probes are shown in Table 1. PCR conditions were as follows: pre-denaturation stage, 95°C for 5 min; PCR reaction, 95°C for 15 sec and 60°C for 60 sec (40, 60, and 50 cycles for *ACTB*, *KISS1*, and *ESR1*, respectively). The gene expression level of the target gene relative to that of *ACTB* was calculated using the ΔΔCt method.

For the determination of *KISS1*- and *ESR1*-expressing neuron-derived cell clones, qRT-PCR of *KISS1*, *ESR1*, and *ACTB* was performed once in triplicate using the P0 cDNA of the 48 neuron-derived cell clones as a template. Averages of the three *KISS1* and *ESR1* Ct values were compared with that of the three *ACTB* Ct values to calculate relative *KISS1* and *ESR1* mRNA expression levels (ΔΔCt method).

The effect of E_2 on the KISS1 expression in the goat POA kisspeptin neuron cell line candidates

Three *KISS1*-positive and *ESR1*-positive cell clones (GP34, GP64, and GP66), selected from the qRT-PCR results, were treated with E_2 (17β-estradiol; #E8875, Sigma-Aldrich, St Louis, MO, USA), and the *KISS1* expression levels were examined. GP34, GP64, and GP66 cells were plated on poly-L-lysine-coated 6-well plates (AGC Techno Glass) at a density of 1.5×10^5 cells/well (GP34 and GP64 cells) or 2.5×10^5 cells/well (GP66 cells) and cultured for 24 h. Vehicle (99.5% ethanol) or E_2 (1, 10, 50, or 100 pM) was added to the culture medium. These E_2 concentrations were based on the physiological range of circulating estrogen in goats: 1.5–9 pg/ml (5–30 pM) in the luteal phase and 20–30 pg/ml (70–110 pM) in the follicular phase [44, 45]. After the 24-h culture, total RNA was extracted, and the *KISS1* expression level was measured by qRT-PCR with the condition mentioned above. The qRT-PCR was performed in triplicate with each cDNA obtained from 3–7 independent experiments. Averages of the three *KISS1* Ct values were compared to those of the three *ACTB* Ct values to calculate the relative *KISS1* mRNA expression levels (ΔΔCt method). Moreover, means and SEM of the *KISS1* expression levels of all the independent experiments were calculated.

Table 1. Primer and probe sequences and PCR conditions for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Gene	Accession no.		Sequence (5'→3')	Annealing temperature (°C)	Cycle	Product size (bp)
KISS1	NM_001285710.2	P	CGCCACCTTTTCCAAGGTCTCCCTGAAG	60	60	83
		F	TGCTCTTCCTTTGTGCCACC			
		R	CTGCGAGCCTGTGGTTCTAG			
ESR1	XM_018053363.1	P	TTGCTGGCTACATCGTCTCGGTTCCGT	60	50	133
		F	AGGCATGGTGGAGATCTTTGAC			
		R	GGACAGAAATGTGTACACTCCAGA			
ACTB	NM_001314342.1	P	CAGGATGCCTCTCTTGCTCTGAGCCTCAT	60	40	105
		F	GCGTGATGGTGGGCATGG			
		R	TGACGATGCCGTGCCCAA			

P, probe; F, forward primer; R, reverse primer.

Immunocytochemistry for kisspeptin and ERα in a goat POA kisspeptin neuron cell line candidate, GP64 cells

The GP64 cells, whose *KISS1* expression was significantly increased by E_2, were examined for the peptide/protein expression of kisspeptin and ERα. The GP64 cells were placed on poly-L-lysine-coated 24-well plates at a density of 3.0×10^4 cells/well and cultured for 48 h. After being washed with Neurobasal medium, the cells were fixed with 99.8% methanol at –20°C for 5 min. After being washed with 0.05 M phosphate-buffered saline (PBS) three times, the cells were incubated with PBS containing 0.2% Triton X-100 (Sigma-Aldrich) (PBST) for 1 h at room temperature. The cells were incubated with a blocking solution consisting of PBST containing 20% heat-inactivated FBS and 1% bovine serum albumin (BSA) (Roche Diagnostics) for 2 h at room temperature. Next, the cells were incubated with anti-kisspeptin polyclonal antibodies (gC2) (dilution 1:500; developed and donated by Dr. Okamura H., Division of Animal Breeding and Reproduction Research, Institute of Livestock and Grassland Science, NARO [46]; RRID: AB_2832253) or anti-ERα polyclonal antibodies (dilution 1:150; #ab3575; Abcam, Cambridge, UK; RRID: AB_303921) in PBST containing 2% FBS and 1% BSA overnight at 4°C. After being washed with PBS three times, the cells were incubated with Alexa 488-conjugated anti-rabbit immunoglobulin (dilution 1:200; #A-11034; Thermo Fisher Scientific; RRID: AB_2576217) for 1.5 h at room temperature. After being washed with PBS three times, the cells were incubated with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) in PBST (dilution 1:1000) for 5 min at room temperature. After being washed with PBST twice, the cells were covered with ProLong Gold Antifade Reagent (Thermo Fisher Scientific). Immunofluorescence was observed under a fluorescent microscope (Olympus, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera (Olympus).

The specificity of the kisspeptin antibody for goat kisspeptin was previously confirmed by an absorption test [46]. To confirm the specificity of the ERα antibody for goat ERα, an absorption test was conducted in the present study by incubating the antibody with an equal weight of human ERα partial peptide (#ab5847; Abcam) overnight at 4°C before the incubation with cells.

Determination of cell growth rates in GP64 cells

GP64 cells were examined for cell growth rates through three passages (P4, P5, and P6). GP64 cells were plated on 12 dishes of poly-L-lysine-coated 35-mm dishes (AGC Techno Glass) at the density of 1.0×10^4 cells/dish. Immediately after the plating, cell numbers of three dishes were counted (day 0). The remaining nine dishes were cultured for 2, 4, and 7 days and cell numbers of three dishes for each day were counted (day 2, 4, and 7, respectively). Means ± SEM of the cell numbers of three dishes in each day and passage were calculated.

RT-PCR

The mRNA expression in a candidate POA kisspeptin neuron-derived cell line (GP64) of P2–P4 was investigated by RT-PCR using the Takara Blend Taq polymerase (Takara Bio) and primers with PCR conditions as shown in Table 2. The expression of *PGR* (progesterone receptor gene) mRNA was examined as a POA kisspeptin neuron marker. The expressions of *TAC3* (neurokinin B gene), *PDYN* (prodynorphin gene), and *TACR3* (tachykinin receptor 3 gene) mRNA were examined as KNDy neuron markers to evaluate if the current cell line candidates were different from the KNDy neurons *in vivo* and a goat KNDy neuron cell line (GA28) [43]. The *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase gene) mRNA expression was examined as a reference gene.

Effect of KCl challenge on the intracellular calcium levels ($[Ca^{2+}]_i$) in GP64 cells

The intracellular calcium levels ($[Ca^{2+}]_i$) in GP64 cells were measured by Ca^{2+} imaging. GP64 cells were seeded on poly-L-lysine-coated cover glasses (Matsunami glass, Osaka, Japan) at 6,000 cells/glass and cultured for 48 h. The cells were loaded with 10 μM fura-2/AM (Dojindo, Kumamoto, Japan) and incubated at 37°C in CO_2 for 30 min. The cover glass with the fura-2-load cells were mounted on an imaging/perfusion chamber with Ringer's solution (140 mM NaCl, 5.6 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 2 mM sodium pyruvate, 9.4 mM glucose, and 5 mM HEPES), and the cells were analyzed for fura-2 fluorescence. Fura-2 fluorescence at 510 nm by excitation at 340 or 380 nm was measured by an inverted fluorescent microscope (Nikon Instech, Tokyo, Japan) and

Table 2. Primer sequences and PCR conditions for real-time reverse transcription-polymerase chain reaction (RT-PCR)

Gene	Accession no.		Primer sequence (5'→3')	Annealing temperature (°C)	Cycle	Product size (bp)
PGR	XM_018059880.1	F	GCATGTCAGTGGACAGATGC	60	40	281
		R	CTCCTTTTTGTCTCAAACCA			
TAC3	NM_001287572.1	F	TGGATGGATTGCTCAAGATG	60	40	129
		R	GAGTGTCTGGCTGGAGGTTC			
PDYN	XM_013968690.2	F	CAGGCCCTACTTGAAGGAACTA	60	40	228
		R	TAACGTTTGACCTGCTCCTTGG			
TACR3	XM_018049248.1	F	CGCAACCAAGATTGTCATTG	60	40	231
		R	CAGAGGGTGATTCCAACGAT			
GAPDH	XM_005680968.3	F	AGTTCCACGGCACAGTCAAG	58	35	474
		R	CGCCAGTAGAAGCAGGGATG			

F, forward primer; R, reverse primer.

an ICCD camera (Hamamatsu Photonics, Shizuoka, Japan). Images were recorded at 2 sec intervals and analyzed by AquaCosmos software (Hamamatsu Photonics). After the perfusion with Ringer's solution for 10 min, 145.6 mM KCl (high KCl) was administered to the cells for 2 min. $[Ca^{2+}]_i$ in each cell was calculated from the ratio of 340/380 nm fluorescence (F ratio). The mean F ratio for 2 min before the start of KCl infusion was calculated as the baseline. The maximum F ratio during the KCl infusion was defined as the peak. $[Ca^{2+}]_i$ was measured in 55 cells and mean baselines and mean peaks during the KCl infusion of the cells were calculated.

Statistical analysis

Data of the *KISS1* expression levels in GP34, GP64, and GP66 cells treated with E_2 were tested for normality using the Shapiro-Wilk test, and then, was analyzed using the Steel's test. The baseline $[Ca^{2+}]_i$ and peak $[Ca^{2+}]_i$ during KCl infusion were statistically compared by a paired t-test. Statistical analyses were performed using R software version 3.5.2. P values less than 0.05 were considered significant.

Results

Selection of goat POA kisspeptin neuron-derived cell clones

qRT-PCR analysis showed that 12 out of the 48 neuron-derived cell clones expressed both *KISS1* and *ESR1*, which are POA kisspeptin neuron markers. Three out of the 12 clones (GP34, GP64, and GP66), in which the *KISS1* and *ESR1* levels were relatively high and stable, were selected for the following experiments. The *KISS1* and *ESR1* levels in the three candidate cell clones (GP34, GP64, and GP66) of P0 are shown in Fig. 1A. Neurite-like structures were observed in GP34, GP64, and GP66 cells (Fig. 1B).

The effect of E_2 on KISS1 expression in GP34, GP64, and GP66 cell clones

The effect of E_2 on the *KISS1* expression level in the three cell clones (GP34, GP64, and GP66) was examined by qRT-PCR. The *KISS1* levels in GP64 cells treated with 1 pM or 10 pM E_2 were significantly higher compared with the vehicle-treated control ($P < 0.01$ or $P < 0.05$, respectively) (Fig. 2). The mean *KISS1* levels

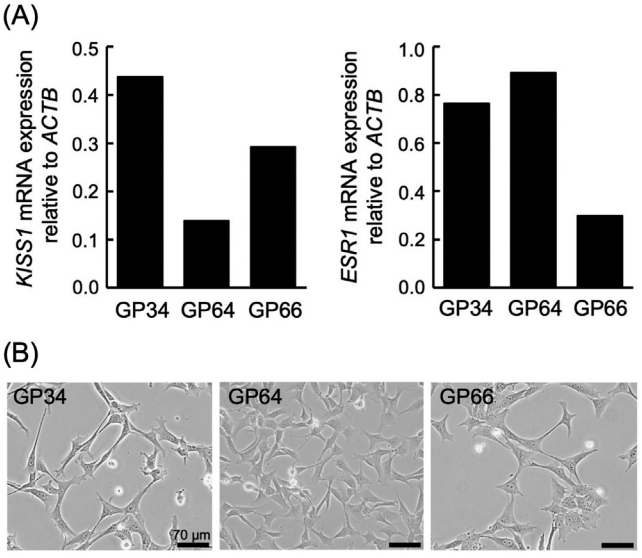

Fig. 1. Kisspeptin gene (*KISS1*) and estrogen receptor α gene (*ESR1*) mRNA expression and morphology of candidate preoptic area (POA) kisspeptin neuron-derived cell clones, GP34, GP64, and GP66. (A) *KISS1* and *ESR1* expression levels in GP34, GP64, and GP66 cells. The *KISS1* and *ESR1* expression levels in GP34, GP64, and GP66 cells of P0 were measured by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) in triplicate and means of *KISS1* and *ESR1* levels relative to actin beta gene (*ACTB*) are shown. (B) Morphology of GP34, GP64, and GP66 cells. Neurite-like structures were observed in all the cell clones. Scale bars, 70 μm.

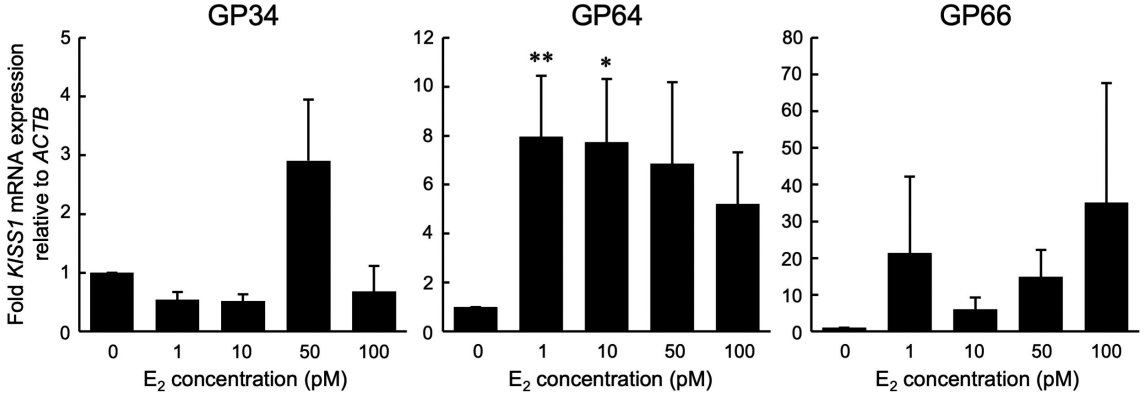

Fig. 2. The effect of 17β-estradiol (E_2) on kisspeptin gene (*KISS1*) mRNA expression in *KISS1*- and estrogen receptor α gene (*ESR1*)-positive goat preoptic area (POA)-derived cell clones, GP34, GP64, and GP66. Cells were cultured with vehicle (0 pM) or E_2 (1, 10, 50, 100 pM) for 24 h and the *KISS1* expression levels were measured by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The mRNA levels were normalized with those of an internal control, actin beta gene (*ACTB*), and represented as relative values to those of the vehicle-treated group. Values are means ± SEM obtained from 3 (GP34), 7 (GP64), and 5 (GP66) independent experiments. Asterisks indicate statistical difference (** $P < 0.01$, * $P < 0.05$ *vs*. vehicle, Steel's test).

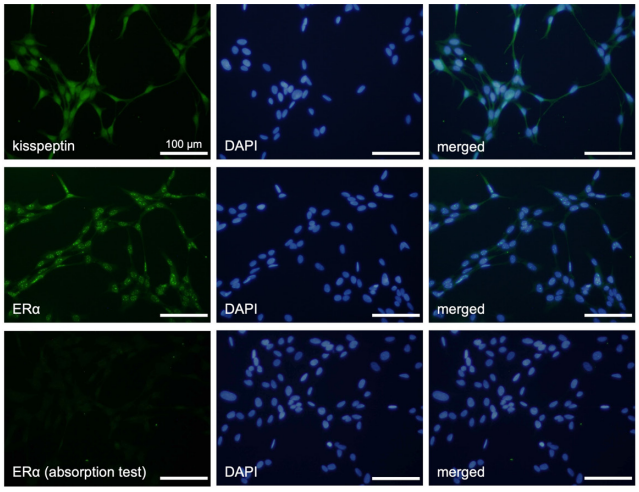

Fig. 3. Peptide/protein expression of kisspeptin and estrogen receptor α (ERα) in GP64 cells examined by immunocytochemistry. The GP64 cells showed immunoreactivities for kisspeptin (upper, left) and ERα (middle, left). The immunoreactivity for ERα was eliminated by the preincubation of the anti-ERα antibody with ERα peptide (absorption test) (bottom, left). The images of nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (middle panels) and merged images of immunoreactivities for kisspeptin and ERα and the nuclear staining (merged) (right panels) are also shown. Scale bars, 100 μm.

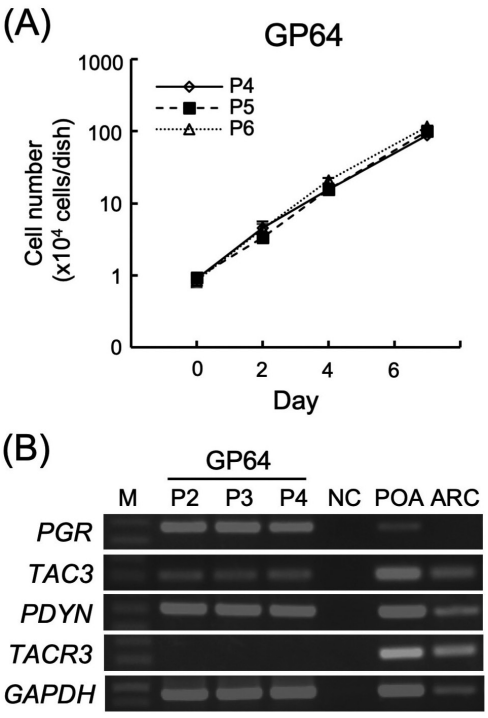

Fig. 4. Growth rates and gene expressions in GP64 cells. (A) The growth rates of GP64 cells from passage 4–6 (P4–P6) are examined. Cells of each passage were plated at 1×10^4 cells/dish and cell numbers were counted at day 0, 2, 4, and 7 after the plating. Means ± SEM of the cell numbers of three dishes are shown. (B) The expression of progesterone receptor gene (*PGR*), neurokinin B gene (*TAC3*), prodynorphin gene (*PDYN*), and tachykinin receptor 3 gene (*TACR3*) in GP64 cells. Representative photographs showing *PGR*, *TAC3*, *PDYN*, *TACR3*, and glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) expression in GP64 cells and goat preoptic area (POA) and arcuate nucleus (ARC) tissues determined by RT-PCR. M, 100 bp DNA ladder marker; NC, negative control (PCR without template).

in GP64 cells treated with 50 pM or 100 pM E_2 were higher than those in the control, but the changes were not significant. In GP34 and GP66 cells, the *KISS1* expression levels were not significantly changed by any E_2 concentrations (Fig. 2).

Peptide/protein expression of kisspeptin and ERα in GP64 cells

The results obtained by an immunocytochemistry for kisspeptin and ERα in representative GP64 cells are shown in Fig. 3. The immunoreactivities of kisspeptin (upper, left) and ERα (middle, left) were detected in GP64 cells. The kisspeptin- and ERα-immunoreactivities were merged with the signal of DAPI (right panels). The immunoreactivity of kisspeptin was detected in the entirety of the cell while that of ERα was detected mainly in the cell nucleus. No immunoreactivity of ERα was found in the cells subjected to the absorption test, indicating the specificity of anti-ERα (bottom panels).

Stable growth rates through GP64 cells passages

The growth rates of GP64 cells through three passages (P4, P5, and P6) are shown in Fig. 4A. GP64 cells of P4, P5, and P6 showed stable growth rates for 7 days.

Gene expression of PGR, TAC3, PDYN, and TACR3 in GP64 cells

PGR, *TAC3*, and *PDYN* expressions were detected in GP64 cells by RT-PCR (Fig. 4B). The expression levels of the genes were stable through three passages (P2, P3, and P4). *TACR3* was not detected through the passages (Fig. 4B).

Effect of KCl challenge on $[Ca^{2+}]_i$ in GP64 cells

No changes in $[Ca^{2+}]_i$ were observed during the perfusion with Ringer's solution for 10 min, while the KCl challenge induced transient increase of $[Ca^{2+}]_i$ (F ratio) in GP64 cells (Fig. 5A). The peak F ratio during the KCl infusion was significantly higher than the baseline F ratio ($P < 0.01$) (Fig. 5B).

Discussion

In this study, goat POA-derived cell clones (GP34, GP64, and GP66) expressing both *KISS1* and *ESR1*, which mimic the gene expression pattern of endogenous POA kisspeptin neurons, were newly generated. One cell clone, GP64, especially showed a significant increase in *KISS1* gene expression in response to E_2 treatment, suggesting that the cell clone shares common characteristics of *in vivo* POA kisspeptin neurons. Importantly, the response of GP64 cells to E_2 was opposite to that of GA28 cells, a goat KNDy neuron cell line, whose *KISS1* expression was significantly suppressed by E_2 treatment [43]. In addition, the GP64 cells showed an expression of

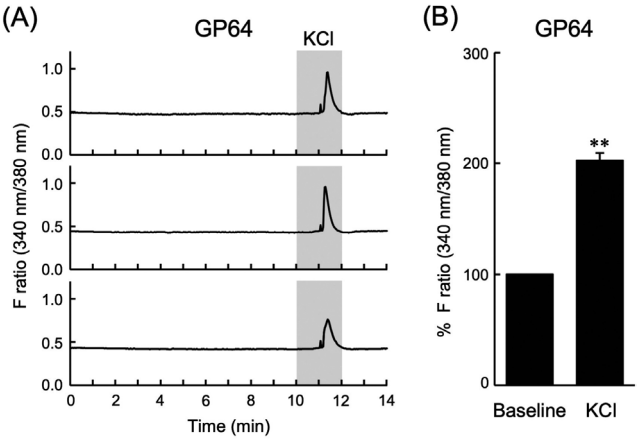

Fig. 5. Intracellular calcium concentration ($[Ca^{2+}]_i$) in GP64 cells treated with high KCl. (A) Changes in $[Ca^{2+}]_i$, measured as F ratio (the ratio of 340/380 nm fura-2 fluorescence), of three representative cells during 0–14 min from the start of recording. Shaded area indicates the period of 145.6 mM KCl administration (10–12 min). (B) Effect of high KCl on $[Ca^{2+}]_i$ in GP64 cells. Values are means ± SEM of the $[Ca^{2+}]_i$ before KCl treatment (Baseline) and the peak $[Ca^{2+}]_i$ during the treatment (KCl) in 55 cells. Asterisks indicate statistical difference (** $P < 0.01$ *vs*. baseline, paired *t*-test).

kisspeptin and ERα at the peptide/protein level. Further, their growth capacity was stable even after three passages, and KCl challenge increased the intracellular calcium levels in the GP64 cells, indicating their neurosecretory ability. Thus, GP64 cells would be a useful model to examine the molecular and cellular mechanisms mediating estrogen positive feedback in POA kisspeptin neurons in ruminants. Thus, the current ruminant POA-derived kisspeptin neuron cell line, GP64, would contribute in investigating the mechanisms responsible for ovulatory disorders in female ruminants, which are important domestic animals, as well as women.

The POA/AVPV kisspeptin neurons are indicated to be direct targets of estrogen positive feedback [29, 30, 35, 47, 48], which is one of the most important characteristics of the neurons to induce GnRH/LH surge in female mammals. Previous *in vivo* studies have demonstrated that E_2 treatment increased *KISS1* expression in AVPV kisspeptin neurons in rodents [28, 31]. In cyclic sheep, the number of *KISS1* cells in the POA was higher in the late follicular phase than in the luteal phase, suggesting that the preovulatory level of E_2 increases *KISS1* expression in POA kisspeptin neurons [12]. The present study showed that *KISS1* expression in GP64 cells was significantly increased in response to the addition of 1 and 10 pM E_2 in the medium, suggesting that the GP64 cells would be the appropriate cell line for analyzing the estrogen positive feedback system. More specifically, 1 or 10 pM E_2 increased *KISS1* expression nearly 8 times as high as the vehicle did in GP64 cells. The response of GP64 cells to the E_2 is consistent with that of the previously established mouse AVPV kisspeptin neuron cell line (KTaV-3), in which the *KISS1* expression increased to 3.8–9.1 times by the treatment with 5–25 pM E_2 [41]. As for another two mice AVPV kisspeptin neuron cell lines (mHypoA-50 and mHypoA-Kiss/GFP-4), *KISS1* expression in the mHypoA-50 increased to at most 1.8 times by 100 nM E_2, and the mHypoA-Kiss/GFP-4 increased to at most 1.5 times by 10 nM E_2 [42]. Thus, GP64 cells showed equivalent or stronger responsiveness to E_2 compared with the mouse AVPV kisspeptin neuron cell lines.

The mice AVPV kisspeptin neuron cell lines increased *Kiss1* expression by the treatment with both the proestrus level of E_2 (25–50 pM [49, 50]) and the lower E_2 such as 5 and 10 pM [41]. Interestingly, the effective E_2 concentrations, 1 and 10 pM, to increase *KISS1* expression in GP64 cells are equivalent to the plasma concentration in Shiba goats of the luteal phase, which are reported to be approximately 1.5–9 pg/ml (5–30 pM), and much lower than the E_2 concentration in the preovulatory phase of cycling Shiba goats, which are approximately 20–30 pg/ml (70–110 pM) [44, 45]. This suggests that the sensitivity to E_2 is likely to be higher in GP64 than in POA kisspeptin neurons *in vivo*. The previous study using rats reported that the treatment with the diestrous level of E_2 significantly increased the number of *Kiss1*-positive cells in the AVPV as well as the preovulatory level of E_2 [28]. Therefore, POA kisspeptin neurons in ruminants might also have an ability to respond to the negative feedback level E_2 as rats do. In this context, the characteristic of GP64 cells to respond to low E_2 is consistent with that of rat AVPV kisspeptin neurons *in vivo*, suggesting the usefulness of GP64 cells as a cellular model for ruminant POA kisspeptin neurons.

Along with *KISS1* and *ESR1* mRNA expression, kisspeptin and ERα were detected at the peptide/protein level in GP64 cells by immunocytochemistry. The distribution pattern of ERα, which is a nuclear receptor, was adequate, as its immunoreactivity was found in the cell nucleus. On the other hand, kisspeptin seemed to be located both in the cytoplasm and nucleus in GP64 cells. Generally, the distribution of neuropeptides such as kisspeptin, neurokinin B, dynorphin A, and GnRH are limited in the cytoplasm when those neurons in brain tissue sections are immunostained. In our previous report, kisspeptin, neurokinin B, and dynorphin A immunoreactivities, and GnRH immunoreactivity were detected in the entirety of the cell in the goat KNDy neuron cell line (GA28) and goat GnRH neuron cell lines (GP11 and GP31), respectively [43]. In addition, immortalized mouse GnRH neuron cell lines showed similar distributions of GnRH: both cytoplasm and nucleus were stained by immunocytochemistry [51]. Molecules of intermediate size (approximately 10–70 kDa), such as enhanced green fluorescent protein (EGFP, 27 kDa), are reported to cross the nuclear envelope, and are located both in the cytoplasm and nucleus in cultured cell lines [52]. Therefore, these neuropeptides, which have relatively small molecular weights (e.g., 16 kDa in kisspeptin), may easily transfer to the nucleus in the goat kisspeptin and GnRH neuron cell lines. However, the cause of the difference in the distribution of kisspeptin in our goat immortalized cell lines from that in *in vivo* kisspeptin neurons is unknown, and needs to be determined.

The current analysis in Ca^{2+} imaging revealed that GP64 cells can be activated by high concentration of KCl, which is an inducer of cellular depolarization, suggesting that GP64 cells are equipped with a functional neurosecretory response system. The $[Ca^{2+}]_i$ response pattern to the KCl was consistent with the previous reports showing the response of an immortalized mouse hypothalamic neuronal cell line (N-38) to high KCl [53], and that of an immortalized mouse GnRH neuron cell line (GT1-1) to high K^+ [54]. The measurement

of intracellular Ca^{2+} concentration of GP64 cells would be useful for screening upper stimulator(s) that regulate the POA kisspeptin neuron activity. It is also expected that GP64 cells can be used for various *in vitro* experiments including gene expression analyses and Ca^{2+} imaging, which may provide easier and more simplified experimental models to evaluate the function of POA kisspeptin neurons than *in vivo* studies using goats, sheep, or cattle.

The present study showed that *PGR* expression was evident in the GP64 cells as detected in the goat POA tissue. This result is consistent with previous reports, showing that *PGR* is expressed in AVPV kisspeptin neurons in mice [55]. Unexpectedly, the current GP64 cells showed *TAC3* and *PDYN* expressions, identical to that of a goat KNDy neuron cell line (GA28) [43]. The previous studies showed that neurokinin B and dynorphin A immunoreactivities were detected in ARC kisspeptin neurons [24–27], but not in POA/AVPV kisspeptin neurons [24–26] in mature female ruminants as well as rodents, suggesting that *TAC3* and *PDYN* are marker genes for KNDy neurons *in vivo*. Thus, there exists a discrepancy between the current immortalized cell line and the previous *in vivo* studies. The present immortalization of the cells might have triggered the expression of those genes, or fetal POA kisspeptin neurons may have different gene expression patterns from those in adults: *KISS1*- and *ESR1*-expressing neurons in the POA of goat fetuses may coexpress *TAC3* and *PDYN*, though the *TAC3*/neurokinin B or *PDYN*/dynorphin A expression in fetal POA kisspeptin neurons are still undetermined in ruminants as well as rodents. Thus, the different characteristics of GP64 cell line from POA kisspeptin neurons in mature animals should be taken into consideration when used for experiments. *TACR3*, a gene encoding the tachykinin receptor 3 (a receptor for neurokinin B), is also known as a gene expressed in KNDy neurons in sheep [56] and mice [26] and is detected in GA28 cells [43]. Unlike GA28 cells, *TACR3* was not detected in GP64 cells, though it remains unclear whether POA/AVPV kisspeptin neurons express *TACR3* or not.

In summary, we newly generated a goat POA-derived immortalized cell line, GP64, which expresses kisspeptin and ERα both at the mRNA and peptide/protein levels and responds to estrogen, similar to endogenous POA kisspeptin neurons, which mediate estrogen positive feedback and regulate GnRH/LH surge. To our knowledge, this is the first report of ruminant POA kisspeptin neuron model. Further studies using this cell line would promote the elucidation of estrogen positive feedback mechanism as well as ovulation induction mechanism in female mammals and contribute to uncover the mechanism responsible for ovulatory disorders in ruminants.

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