Anti-Müllerian hormone receptor type 2 is expressed in gonadotrophs of post-pubertal heifers to control gonadotropin secretion

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Running head: AMHR2 controls gonadotropin secretion

Abstract. Preantral and small antral follicles may secret anti-Müllerian hormone (AMH) to control gonadotropin secretion from ruminant gonadotrophs. This study investigated whether the main receptor for AMH, AMH receptor type 2 (AMHR2), is expressed in gonadotrophs of post-pubertal heifers to control gonadotropin secretion. RT-PCR detected expressions of AMHR2 mRNA in anterior pituitaries (APs) of post-pubertal heifers. We developed an anti-AMHR2 chicken antibody against the extracellular region near the N terminus of bovine AMHR2. Western blotting utilizing this antibody detected the expressions of AMHR2 protein in APs. Immunofluorescence microscopy utilizing the same antibody visualized colocalization of AMHR2 with gonadotropin-releasing hormone (GnRH) receptor on the plasma membrane of gonadotrophs. We cultured the AP cells for 3.5 days, and then treated them with increasing concentrations (0, 1, 10, 100, or 1000 pg/ml) of AMH. AMH (10–1000 pg/ml) stimulated ($P < 0.05$) basal FSH secretion. The hormone (100–1000 pg/ml) also stimulated ($P < 0.05$) basal LH secretion weakly. However, AMH (100–1000 pg/ml) inhibited GnRH-induced FSH secretion, but not GnRH-induced LH secretion, in AP cells. In conclusion, AMHR2 is expressed in gonadotrophs of post-pubertal heifers to control gonadotropin secretion.

Additional keywords: AMHR2, GnRH receptor, Müllerian-inhibiting substance, ruminant.

34

35 **Short summary**

36 This study revealed that gonadotrophs express the receptor for anti-Müllerian hormone
37 (AMH) in post-pubertal heifers, and the AMH receptor colocalized with gonadotropin-
38 releasing hormone receptors on the surface of gonadotrophs. Furthermore, AMH
39 stimulated gonadotropin secretion from anterior pituitary cells of post-pubertal heifers.
40 Therefore, preantral and small antral follicles may secret AMH to control the
41 gonadotropin secretion from gonadotrophs in post-pubertal heifers.

42

Introduction

Gonadotrophs in the anterior pituitaries (APs) secrete gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), to regulate follicle growth, ovulation, and corpus luteum formation in ovaries of vertebrates. Acting as a feedback mechanism, antral follicles and corpora lutea secrete steroids and inhibin to control gonadotropin secretion from the AP (Martin *et al.* 1991). This pituitary-ovary axis is one of the most important fundamental mechanisms for reproduction. However, it is not clear whether hormones secreted from preantral and small antral follicles control gonadotropin secretion from the AP. We have a question whether preantral and small antral follicles are silent majority in ovaries.

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein in the transforming growth factor (TGF)-β family, and AMH is produced mainly by granulosa cells of the preantral and small antral follicles in humans and animals (Bhide *et al.* 2016). AMH regulates follicular development during the gonadotropin-responsive phase (Hernandez-Medrano *et al.* 2012) and to inhibit follicular atresia (Seifer *et al.* 2014). Blood AMH concentrations are indicative of ovarian aging in women (Bhide *et al.* 2016; Dewailly *et al.* 2014). Plasma AMH concentrations positively correlate with pregnancy rates in dairy cows (Ribeiro *et al.* 2014). Further, circulating AMH concentrations can predict the

number of high-quality embryos produced by a donor goat or cow (Ireland *et al.* 2008; Monniaux *et al.* 2011). These data suggest the importance of AMH for proper reproductive function in ruminants after puberty.

Although the primary role of AMH is at the ovary level in female animals, AMH secreted from preantral and small antral follicles into circulating blood may have roles in other organs. Indeed, the APs of adult rats express mRNA for the main receptor of AMH, AMH receptor type 2 (AMHR2) (Bédécarrats *et al.* 2003). AMH activates LHβ and FSHβ gene expression in LβT2 cells—a murine gonadotroph-derived cell line (Bédécarrats *et al.* 2003). Garrel *et al*. (2016) recently reported that AMH stimulates FSH secretion in rats *in vivo*; however, such stimulation is restricted to pre-pubertal female rats. However, there are still no data on the regulatory role of AMH on gonadotropin secretion from gonadotrophs in ruminant species.

Gonadotrophs are controlled by GnRH *via* the GnRH receptor (GnRHR) that are present in lipid rafts in the plasma membrane of gonadotrophs (Navratil *et al.* 2009; Wehmeyer *et al.* 2014; Kadokawa *et al.* 2014). The lipid rafts are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons *et al.* 2000; Head *et al.* 2014), and they facilitate signaling by allowing colocalization of membrane receptors and their downstream signaling components

(Simons *et al.* 2000; Head *et al.* 2014). We recently discovered that two orphan receptors, GPR61 and GPR153, are colocalized with GnRHR in gonadotroph plasma membrane lipid rafts (Pandey *et al.* 2017a, 2017b). Therefore, gonadotroph lipid rafts containing GnRHR may contain AMHR2. In the present study, we tested the hypothesis that AMHR2 is expressed in the gonadotrophs of post-pubertal heifers to control gonadotropin secretion.

Materials and Methods

AP and ovary sample collection

We obtained AP tissue from post-pubertal (26 months of age) Japanese Black heifers at a local abattoir, using a previously described method (Kadokawa *et al.* 2014). The heifers were in the middle luteal phase, i.e., 8 to 12 days after ovulation, as determined by macroscopic examination of the ovaries and uterus (Miyamoto *et al*. 2000); the AP show the highest LH and GnRHR concentrations in this phase (Nett *et al.* 1987).

Granulosa cells in small antral follicles express AMHR2 mRNA (Poole *et al.* 2016). Therefore, we also collected ovary tissue samples from the same heifers to use as positive controls of AMHR2 in western blotting and immunohistochemistry assays.

The AP and ovary samples for RNA or protein (n = 3) extraction were immediately

97 frozen in liquid nitrogen and stored at −80°C. The AP and ovary samples for
98 immunohistochemistry (n = 5) were fixed with 4% paraformaldehyde at 4°C for 16 h.
99 The AP samples meant for cell culture followed by immunocytochemical analysis (n = 5)
100 and those that were to be used for cell culture to evaluate the effect of AMH on LH and
101 FSH secretion (n = 8) were stored in ice-cold 25 mM HEPES buffer (pH 7.2) containing
102 10 mM glucose and transported on ice to the laboratory.
103
104 *RT-PCR, sequencing of amplified products, and homology search in gene databases*
105 Total RNA was extracted from the AP samples (n = 3) using RNAiso Plus (Takara
106 Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The extracted RNA
107 samples were treated with ribonuclease-free deoxyribonuclease (Toyobo, Tokyo, Japan)
108 to eliminate possible genomic DNA contamination. Using a NanoDrop ND-1000
109 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), the
110 concentration and purity of each RNA sample were evaluated to ensure the A_{260}/A_{280} nm
111 ratio was in the acceptable range of 1.8–2.1. The mRNA quality of all samples was
112 verified by electrophoresis of total RNA followed by staining with ethidium bromide, and
113 the 28S:18S ratios were 2:1. The cDNA was synthesized from 0.5 µg of the total RNA

per AP using ReverTra Ace qPCR RT Master Mix (Toyobo) according to the manufacturer's protocol.

In order to determine the expression of AMHR2 mRNA in the AP, PCR was conducted using one of three pairs of primers designed by Primer3 based on reference sequence of bovine AMHR2 [National Center for Biotechnology Information (NCBI) reference sequence of bovine AMHR2 is NM_001205328.1], as one of PCR primers must span exon-exon junction. Table 1 shows the details of the primers, and the expected PCR-product sizes of the AMHR2 were 340 bp, 320 bp, and 277 bp. Using a Veriti 96–Well Thermal Cycler (Thermoscientific), PCR was performed using 20 ng of cDNA and polymerase (Tks Gflex DNA Polymerase, Takara Bio Inc.) under the following thermocycles: 94 °C for 1 min for pre-denaturing followed by 35 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. PCR products were separated on 1.5% agarose gel by electrophoresis with a molecular marker [Gene Ladder 100 (0.1-2kbp), Nippon Gene, Tokyo, Japan], stained with fluorescent stain (Gelstar, Lonza, Allendale, NJ), and observed using a charge-coupled device (CCD) imaging system (GelDoc; Bio-Rad, Hercules, CA, US). The PCR products were purified with the NucleoSpin Extract II kit (Takara Bio Inc.) and then sequenced with a sequencer (ABI3130, Thermo Fisher Scientific, Waltham, MA, US) using one of the PCR primers and the Dye Terminator v3.1

Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences obtained were used as query terms with which to search the homology sequence in the DDBJ/GenBankTM/EBI Data Bank using the basic nucleotide local alignment search tool (BLAST) optimized for highly similar sequences (available on the NCBI website).

Development anti-AMHR2 chicken antibody

We previously determined using the SOSUI v.1.11 algorithm (Hirokawa *et al.* 1998; http://harrier.nagahama-i-bio.ac.jp/sosui/) that bovine AMHR2 protein [543 amino acids; accession number NP_001192257.1 in NCBI reference bovine sequences] contains one hydrophobic transmembrane domains (amino acid 146–168) linked by hydrophilic extracellular and intracellular regions. This structure is the same as the reported structure of mouse AMHR2 (Sakalar *et al.* 2015).

Genetyx ver. 11 (Gentyx, Tokyo, Japan) was utilized to predict antigenic determinants based on an algorithm derived by Hopp and Woods (1981). For antibody production, a peptide corresponding to amino acids 31–45 (GVRGSTQNLGKLLDA), an extracellular region that is located near the N terminus of the AMHR2, was used for three reasons. First, this peptide has no homology to the corresponding region of chicken AMHR2 (XP_015145444.1). Second, the peptide sequences are in downstream region of the signal

peptide of bovine AMHR2 (amino acid 1–17). Third, we could confirm that no other protein encoded in the bovine genome exhibited homology to the peptide sequences of the AMHR2 by comparison with the sequences retrieved from DDBJ/GenBankTM/EBI Data Bank, using the protein BLAST.

A commercial service (Scrum Inc., Tokyo, Japan) was utilized for synthesis of antigen peptide (C-GVRGSTQNLGKLLDA), conjugation with keyhole limpet hemocyanin (KLH), immunization, and antibody purification. Briefly, the peptide was synthesized and the purity verified (>99.0%) using high-performance liquid chromatography followed by mass spectrometry. Then, KLH was conjugated to the sulfhydryl group of the cysteine to produce an immunogen that was then emulsified with Complete Freund's adjuvant and injected into chickens five times at 14-day intervals. Blood was collected 7 days after the final immunization and the antibody was purified by affinity column chromatography (PD10; GE Healthcare, Amersham, UK) containing an antigen-conjugated gel prepared with the SulfoLink Immobilization Kit (Thermo Scientific).

Other antibodies used in this study

We previously developed a guinea pig polyclonal antibody that recognizes the N-terminal extracellular domain (corresponding to amino acids 1–29;

168 MANSDSPEQNENHCSAINSSIPLTPGSLP) of GnRHR (anti-GnRHR). The specificity
169 of the anti-GnRHR antibody was verified by western blotting, and pretreatment with anti-
170 GnRHR antibody inhibited GnRH-induced LH secretion from cultured bovine
171 gonadotroph (Kadokawa *et al*. 2014). Additionally, we previously used the anti-GnRHR
172 antibody for immunofluorescence detection of GnRHR in plasma membrane of bovine
173 gonadotroph (Kadokawa *et al*. 2014; Pandey *et al.* 2016). We observed a strong and
174 localized GnRHR-positive staining signal as aggregation on the plasma membrane of
175 gonadotrophs (Kadokawa *et al.* 2014). We used the anti-GnRHR as well as a mouse
176 monoclonal anti-LH β (LHβ) subunit antibody (clone 518-B7; Matteri *et al.* 1987) for
177 immunohistochemical analysis of AP tissue and cultured AP cells. This antibody does not
178 cross-react with other pituitary hormones (Iqbal *et al.* 2009). We also used a mouse
179 monoclonal anti-FSH β (FSHβ) subunit antibody (clone A3C12) that does not cross-react
180 with other pituitary hormones (Borromeo *et al.* 2004) for immunohistochemical analysis
181 of AP tissue.
182
183 *Western Blotting for AMHR2*
184 Briefly, we extracted protein from the samples of AP (n = 3) or ovary (n = 3, used as
185 positive control) and performed western blotting as previously described (Kadokawa *et*

186 *al.* 2014). The extracted protein (33.4 μg of total protein in 37.5 μl) was mixed in 12.5 μl
187 of 4x Laemmli sample buffer (Bio-rad) containing 10% (v/v) β-mercaptoethanol, then
188 boiled for 3 min at 100 °C. Boiled protein samples were quickly cooled in ice, then 4, 8,
189 or 16 μg of total protein were loaded onto sodium dodecyl sulfate a polyacrylamide gels,
190 along with a molecular weight marker (Precision Plus Protein All Blue Standards; Bio-
191 Rad), for resolution by electrophoresis at 100 V for 90 min. Proteins were then transferred
192 to polyvinylidene fluoride (PVDF) membranes for immunoblotting with the anti-AMHR2
193 chicken antibody (1:25,000 dilution) after blocking with 0.1% Tween 20 and 5% non-fat
194 dry milk for 1 h at 25 °C. The membranes were incubated overnight at 4 °C with the
195 primary antibody, washed with 10 mM Tris–HCl (pH 7.6) containing 150 mM NaCl and
196 0.1% Tween 20, and incubated with horseradish peroxidase (HRP)-conjugated anti-
197 chicken IgG goat antibody (Bethyl laboratories, Inc., Montgomery, TX, USA; 1:50,000
198 dilution) at 25 °C for 1 h. Protein bands were visualized using an ECL-Prime
199 chemiluminescence kit (GE Healthcare) and CCD imaging system (Fujifilm, Tokyo,
200 Japan). Previous studies utilizing western blotting for AMHR2 reported that human and
201 mouse AMHR2 are present as dimers, full-length monomers, or cleaved monomers
202 (Faure *et al.* 1996; Hirschhorn *et al.* 2015). Thus, we defined bovine AMHR2 bands based
203 on mobility as one of these structure types. After antibodies were removed from the PVDF

membrane with stripping solution (Nacalai Tesque Inc., Kyoto, Japan), the membrane was used for immunoblotting with the anti-β-actin mouse monoclonal antibody (A2228, 1:50,000 dilution; Sigma-Aldrich, St. Louis, MO, USA).

Fluorescent immunohistochemistry and confocal microscopic observation

After storage in 4% paraformaldehyde PBS at 4°C for 16 h, the AP (n = 5) or ovary (n = 5) tissue blocks were placed in 30% sucrose PBS until the blocks were infiltrated with sucrose. The methods for immunofluorescence analysis of AP tissue have been described previously (Kadokawa *et al.* 2014). Briefly, we prepared 15-μm sagittal sections and mounted them on slides. The sections were treated with 0.3 % Triton X-100 in PBS for 15 min, then, incubated with 0.5 mL of PBS containing 10% normal goat serum (Wako Pure Chemicals, Osaka, Japan) for blocking for 1 h. Incubation with a cocktail of primary antibodies (anti-GnRHR guinea pig antibody, anti-AMHR2 chicken antibody, and either anti-LHβ or anti-FSHβ mouse antibody [all diluted as 1:1,000]) for 12 h at 4°C was followed by incubation with a cocktail of fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa Fluor 647 goat anti-guinea pig IgG [all from Thermo Fisher Scientific and diluted as 1 μg/mL]) and 1 μg/mL of 4′, 6′-diamino-2-phenylindole (DAPI;

Wako Pure Chemicals) for 2 h at room temperature. Moreover, we prepared 15-μm ovary sections, incubated with anti-AMHR2 chicken antibody (1:1,000), and then incubated with 1 μg/mL Alexa Fluor 488 goat anti-chicken IgG and DAPI to use as positive controls to verify the anti-AMHR2 antibody.

The stained sections on slides were observed with a confocal microscope (LSM710; Carl Zeiss, Göttingen, Germany) equipped with diode (405 nm), argon (488 nm), HeNe (533 nm) and HeNe (633 nm) lasers. Images obtained by fluorescence microscopy were scanned with a 40× or 63× oil-immersion objective and recorded by a CCD camera system controlled by ZEN2012 black edition software (Carl Zeiss). GnRHR, AMHR2, and LHβ or FSHβ localization were examined in confocal images of triple-immunolabeled specimens. In the confocal images obtained after immunohistochemistry analysis, the GnRHR is shown in green, AMHR2 is shown in red, and LHβ or FSHβ is shown in light blue. Therefore, the yellow coloration on the surface of light blue-colored cells indicates the colocalization of AMHR2 and GnRHR. The percentage of AMHR2 single (red)–labeled light blue-colored cells, or the percentage of double (yellow)-labeled light blue-colored cells, among all of the AMHR2-positive light blue-colored cells (sum of the numbers of red-labeled and yellow-labeled light blue-colored cells), were determined from 12 representative confocal images per pituitary gland. Moreover, the

240 percentage of GnRHR single (green)-labeled light blue-colored cells, or the percentage
241 of double (yellow)-labeled light blue-colored cells, among all of the GnRHR-positive
242 light blue-colored cells (sum of the numbers of green-labeled and yellow-labeled light
243 blue-colored cells), were determined from 12 representative confocal images per pituitary
244 gland. To verify the specificity of the signals, we included several negative controls in
245 which the primary antiserum had been omitted or pre-absorbed with 5 nM of the same
246 antigen peptide, or in which normal chicken IgG (Wako Pure Chemicals) was used
247 instead of the primary antibody.
248
249 *AP cell culture and immunocytochemical analysis of cells*
250 The AP cells from 5 heifers were enzymatically dispersed using the method of Suzuki
251 *et al.* (2008), and cell viability was confirmed to be greater than 90% by Trypan blue
252 exclusion. Total cell yield was $19.8 \times 10^6 \pm 0.8 \times 10^6$ cells per pituitary gland. The
253 dispersed cells were then suspended in Dulbecco's Modified Eagle's Medium (DMEM;
254 Thermo Fisher Scientific) containing 1× nonessential amino acids (Thermo Fisher
255 Scientific), 100 U/mL penicillin, 50 μg/mL streptomycin, 10% horse serum (Thermo
256 Fisher Scientific), and 2.5% fetal bovine serum (Thermo Fisher Scientific). The cells
257 (2.5×10^5 cells/mL, total = 0.15 mL per lane) were cultured in the culture medium at

37 °C in 5% CO_2 for 82 h, using a microscopy chamber (μ-Slide VI 0.4, Ibidi, Planegg, Germany). We cultured the AP cells for 82 h (3.5 days), as previously described (Hashizume *et al.* 2003; Kadokawa *et al.* 2008; Hashizume *et al.* 2009; Kadokawa *et al.* 2014; Nakamura *et al.* 2015). We supplied recombinant human activin A (final concentration, 10 ng/ml; R&D systems, Minneapolis, MN, US) to stimulate FSH synthesis at 24 h prior to fixation. Mature activin A of bovines (NP_776788.1) and ovines (NP_001009458.1) have 100% homology with that of humans (CAA40805.1), and the 24 h culture with the same concentration of same recombinant human activin A product stimulates FSH expression in cultured ovine AP cells (Young *et al.* 2008).

We fixed and treated the cultured cells using either 4% paraformaldehyde for 3 min followed by 0.1% Triton X-100 treatment for 1 min (PFA-Triton method), or fixation for 2 min with CellCover (Anacyte Laboratories UG, Kuhreder, Hamburg), instead of 4% PFA, and no Triton X-100 treatment (CellCover method), as described by Kadokawa *et al. (*2014). Briefly, one of the aforementioned methods was used to treat the cells attached to the bottom of the microscopy chamber. For the PFA-Triton method, the fixed cells were incubated with 0.1 mL of the same cocktail of primary antibodies for 2 h at room temperature. Incubation with Triton X-100 allowed both anti-GnRHR and anti-AMHR2 antibodies to bind to target proteins in the cytoplasm and at the cell surface. For the

CellCover method, the fixed cells were incubated with only guinea pig anti-GnRHR and chicken anti-AMHR2 (both 1:1,000) for 2 h at room temperature. The cells were not treated with Triton X-100, so the antibodies bound only to the extracellular domains of the respective receptors in most cells, although some cytoplasmic labeling occurred in broken cells. For both PFA-Triton and CellCover methods, cells were incubated with fluorochrome-conjugated secondary antibody cocktail and DAPI, and subjected to confocal microscopy to produce fluorescence micrographs and differential interference contrast (DIC) images on a single plane. Signal specificity was confirmed using negative controls in which the primary antiserum was omitted or pre-absorbed with 5 nM antigen peptide, or in which the normal chicken IgG replaced the primary antibody. Eight randomly selected images of cells prepared by CellCover method were analyzed for co-localization utilizing the ZEN 2012 black edition software (Carl Zeiss) to calculate overlap coefficients (Manders *et al.* 1993) for the Alexa Fluor 488 and Alexa Fluor 647 fluorophores.

Pituitary cell culture and analysis of the effects of AMH on LH and FSH secretion

The AP cells derived from 8 heifers were prepared using the protocol described above. After the cells (2.5×10^5 cells/mL, total 0.3 mL) had been plated in 48-well culture plates

294 (Sumitomo Bakelite, Tokyo, Japan), they were maintained at 37°C in a humidified
295 atmosphere of 5% CO_2 for 82 h. We supplied the recombinant human activin A (final
296 concentration, 10 ng/ml) to stimulate FSH synthesis at 24 h prior to the AMH test.
297 In the test to evaluate the effect of AMH in the absence of GnRH, the old medium
298 was replaced by 295 μL DMEM containing 0.1% BSA and 10 ng/ml activin A and
299 incubated for 2 h. Treatment was performed by adding 5 μL of DMEM alone or 5 μL of
300 DMEM containing various concentrations of human recombinant AMH (R & D systems;
301 final concentration of 0, 1, 10, 100, or 1000 pg/ml AMH).
302 The bioactive region in the carboxyl-terminal region of mature AMH (Belville *et al.*
303 2004) of bovines (NP_776315.1) and goat (XP_017906255.1) has 96% homology with
304 that of humans (NP_000470.2), and the same recombinant human AMH product shows
305 the biological effect for goat follicles (Rocha *et al.* 2016).
306 After incubation for further 2 h, the medium from each well was collected for
307 radioimmunoassay (RIA) analyses of LH and FSH levels. The physiological
308 concentration of AMH in blood ranged between 5 and 300 pg/ml in Japanese Black cows
309 in our previous study (Koizumi and Kadokawa 2017). Therefore, we used the above-
310 mentioned AMH concentration in this study.
311 In the test to evaluate the effect of AMH in the presence of GnRH, the old medium

312 was replaced by 290 μL DMEM containing 0.1% BSA and 10 ng/ml activin A and
313 incubated at 37ºC for 2 h. Pretreatment was performed by adding 5 μL of DMEM alone
314 or 5 μL of DMEM containing various concentrations (0, 60, 600, 6000, and 60000 pg/ml)
315 of the human recombinant AMH. The cells were incubated while gently shaking for 5
316 min, and then, cells were treated with 5 μL of 60 nM GnRH (Peptide Institute Inc., Osaka,
317 Japan) dissolved in DMEM for 2 h in order to stimulate LH and FSH secretion. The
318 pretreatment plus the GnRH treatment yielded a final concentration of 0, 1, 10, 100, or
319 1000 pg/ml AMH. The final concentration of GnRH was 1 nM in all treatments
320 (Kadokawa *et al.* 2014), except the "control". Control wells were treated with 5 μL of
321 DMEM, but were not incubated with GnRH. "GnRH" wells were pre-treated with 5 μL
322 of DMEM for 5 min and were then incubated with GnRH for 2 h. After incubation for 2
323 h, the medium from each well was collected for LH and FSH RIAs.
324
325 *RIAs to measure gonadotropin concentration in culture media*
326 The concentration of LH was measured in duplicate samples of culture media by
327 double antibody RIA using ^{125}I-labeled bLH and anti-oLH-antiserum (AFP11743B and
328 AFP192279, National Hormone and Pituitary Program of the National Institute of
329 Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, CA, USA). The limit

of detection was 0.40 ng/mL. At 2.04 ng/mL, the intra-assay coefficient of variation was 3.6% and inter-assay coefficient of variation was 6.2%. The concentration of FSH was measured in duplicate samples of culture media by double antibody RIA using ^{125}I-labeled bFSH, reference grade bFSH, and anti-oFSH antiserum (AFP5318C, AFP5346D, and AFPC5288113, NIDDK). The limit of detection was 0.20 ng/mL. At 4.00 ng/mL, the intra-assay coefficient of variation was 4.3% and inter-assay coefficient of variation was 7.1%.

Statistical analysis

The statistical significance of differences in LH or FSH concentration were analyzed by one-factor ANOVA followed by *post-hoc* comparisons using Fisher's protected least significant difference (PLSD) test using StatView version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Expression of AMHR2 mRNA in AP of post-pubertal heifers

The expected PCR products (size 340 bp, 320 bp, and 277 bp) were observed in the

agarose gel after electrophoresis (Fig. 1). Homology searching in the gene databases for the obtained sequence of amplified products using the first, second and third primer pair respectively revealed that the best match alignment was bovine AMHR2 (NM_001205328.1), which had a query coverage of 100%, an e-value of 0.0, and a maximum alignment identity of 99%. No other bovine gene was found to have a homology for the obtained sequences of amplified products, leading to the conclusion that the sequences of the amplified products were identical with the sequence of bovine AMHR2.

Western blotting for AMHR2

The presence of AMHR2 in the AP and ovarian tissue was analyzed by western blot, using anti-AMHR2 antibody (Fig. 2). The anti-AMHR2 antibody revealed similar bands in the two tissues, with few differences (Fig. 2A). The major difference was that AP tissue showed weaker bands than ovarian tissue did. Nevertheless, β-actin bands showed weaker staining in both tissue types (Fig. 2B). Finally, another difference was that the full-length monomer in the ovary appeared as a single band, whereas in AP cells, it appeared as a doublet (Fig. 2A). No bands were observed in the negative control membranes, where the primary antiserum was pre-absorbed with the antigen peptide.

366

367 *Immunofluorescence analysis of AMHR2 expression in bovine granulosa cells*

368 Fig. 3 shows the immunofluorescence in the granulosa cells of small (about 5 mm)
369 follicles in the ovary tissues of post-pubertal heifers. Strong AMHR2 staining appeared
370 to be aggregated, not evenly dispersed.

371

372 *Immunofluorescence analysis of AMHR2 expression in bovine AP tissue*

373 Expression of LHβ, FSHβ, GnRHR, and AMHR2 in bovine AP tissue was
374 investigated by immunohistochemistry (Fig. 4). AMHR2 and GnRHR colocalized in the
375 majority of both LHβ-positive (Fig. 4A) and FSHβ-positive (Fig. 4B) cells. Focus depth
376 of the high magnification lens used in this study are thin, thus, the best focus for GnRHR
377 and AMHR2 on plasma membrane was quite different from both the best focus for
378 nucleus and the best focus for cytoplasmic LHβ or FSHβ. Thus, we could know both
379 membrane receptors are on the cell-surface. Percentages of single- and double-labeled
380 AMHR2- and GnRHR-positive cells were determined from 12 representative confocal
381 images per pituitary gland. In each pituitary gland, there was an average of 52.4 ± 2.4
382 GnRHR-positive cells, 44.6 ± 1.2 AMHR2-positive cells, and 33.6 ± 1.3 double-positive
383 cells; 64.5% ± 3.2% of GnRHR-positive cells were AMHR2-positive, whereas 78.4% ±

384 1.8% of AMHR2-positive cells were GnRHR-positive.

385

386 *AMHR2 and GnRHR aggregate on the surface of cultured AP cells*

387 In the AP cells prepared by the CellCover method, AMHR2 aggregated on the surface
388 of GnRHR-positive cells (Fig. 5). The overlap coefficient between AMHR2 and GnRHR
389 was 0.76 ± 0.05 on the cell surface of cultured AP cells.

390

391 *AMHR2 expression in cultured gonadotrophs*

392 Among the AP cells prepared by the PFA-Triton method, we observed AMHR2 in both
393 LHβ-positive and FSHβ-positive cells (Fig. 6).

394

395 *Effects of AMH on gonadotropin secretion from cultured AP cells*

396 Fig. 7 shows the effect of various concentrations of AMH on LH secretion from the
397 AP cells derived from post-pubertal heifers cultured in the absence (A) or presence (B)
398 of GnRH. In the absence of GnRH (Fig. 7A), 100 pg/ml and 1000 pg/ml of AMH
399 increased ($P < 0.05$) LH secretion, when compared with the controls (17.6 ± 2.4 ng/ml).
400 Conversely, there was no effect of AMH on the GnRH-induced LH secretion (Fig. 7B).

401 Fig. 8 shows the effect of various concentrations of AMH on FSH secretion from the

AP cells derived from post-pubertal heifers cultured in the absence (A) or presence (B) of GnRH. The effect of different concentrations of AMH was significant ($P < 0.05$) in the absence of GnRH (Fig. 8A). The wells with 10 pg/ml ($P < 0.05$), 100 pg/ml ($P < 0.05$), and 1000 pg/ml ($P < 0.05$) of AMH, but not 1 pg/ml of AMH, had higher FSH concentrations than those without AMH (8.4 ± 1.2 ng/ml). The effect of different concentrations of AMH was significant ($P < 0.05$) in the presence of GnRH (Fig. 8B). FSH concentrations in the medium of GnRH wells were higher ($P < 0.05$) than those in the medium of control wells. There was no effect of 1 pg/ml or 10 pg/ml of AMH on the GnRH-induced FSH secretion. There was a suppressing effect of 100 pg/ml ($P < 0.05$) and 1000 pg/ml ($P < 0.05$) of AMH on the GnRH-induced FSH secretion.

Discussion

To the best of our knowledge, this study is the first to report that AP cells express AMHR2 in ruminants and that AMH significantly affects LH and FSH secretion from AP cells. Fluorescent immunohistochemistry using the anti-AMHR2 antibody showed the strong signal located on the surface of granulosa cells in small antral follicles, where AMHR2 mRNA is expressed (Poole *et al.* 2016). Therefore, the anti-bovine AMHR2 is the first developed tool that can be used for immunohistochemistry in bovine samples.

In this study, treatment with 10–1000 pg/ml of AMH stimulated FSH secretion in the absence of GnRH. This agrees with *in vivo* experiments on rats, where AMH stimulates the secretion and expression of FSH (Garrel *et al.* 2016). These data suggested that AMH might bind with AMHR2 to increase FSH secretion from gonadotroph in ruminants as well. Garrel *et al*. (2016) recently reported that AMH increases both FSHβ expression and phosphorylates SMAD 1/5/8 in LβT2 cells, but such increases are blocked by GnRH. In this study, 1–10 pg/ml AMH did not change GnRH-stimulated FSH secretion; however, 100–1000 pg/ml AMH suppressed GnRH-stimulated FSH secretion. Therefore, further studies are required to clarify the molecular mechanisms controlling FSH secretion from ruminant gonadotrophs by AMH and GnRH, especially whether the SMAD 1/5/8 pathways have important roles.

Multiparous (third parity or higher) Japanese Black cows have significantly higher blood AMH concentrations (100 pg/ml level) than primiparous cows (1–10 pg/ml level) throughout the postpartum period (Koizumi and Kadokawa 2017). The multiparous Japanese Black cows have larger number of days from parturition to postpartum first ovulation than the primiparous cows (Koizumi and Kadokawa 2016). Therefore, the suppressing effect of 100–1000 pg/ml of AMH on GnRH-stimulated FSH secretion may

438 have an important role in the follicular growth and delayed postpartum first ovulation in
439 multiparous cows.

440 Intraperitoneal injection with AMH increases FSH concentration in blood collected
441 18 h later, but only in pre-pubertal female rats (Garrel *et al*. 2016). In contrast, this study
442 shows the significant effect of AMH on FSH secretion from the AP of post-pubertal
443 heifers *in vitro*. Therefore, further studies are required to clarify whether there are any
444 differences in AMH effects on FSH secretion among species.

445 The pituitary gland is located outside the blood-brain barrier unlike the hypothalamus
446 (Nussey and Whitehead 2001); therefore, the AMHR2 on gonadotrophs may bind AMH
447 secreted from preantral and small antral follicles. Our data suggested that AMH, like the
448 other TGF-β family members such as inhibin and activin (Kushnir et al. 2017), can affect
449 FSH secretion from gonadotrophs. However, little is known about the changes occurring
450 in the blood AMH concentration during the estrous cycle in ruminants (Pfeiffer *et al*.
451 2014; Koizumi and Kadokawa 2017). The blood AMH concentration is influenced by age
452 and parity (Koizumi and Kadokawa 2017); however, the concentration may not show a
453 considerable change during the estrous cycle in ruminants *in vivo* (Pfeiffer *et al*. 2014;
454 Koizumi and Kadokawa 2017; El-Sheikh Ali *et al*. 2013). Therefore, we must be cautious
455 when concluding that AMH contributes largely in controlling LH and FSH secretion from

456 gonadotrophs *in vivo*.

457 Our results suggested that preantral and small antral follicles may control
458 gonadotropin secretion from the AP in post-pubertal heifers. Conversely, FSH suppresses
459 AMH secretion from bovine granulosa cells (Rico *et al.* 2011). Therefore, there may be
460 feedback mechanisms between gonadotrophs and granulosa cells in preantral and small
461 antral follicles. AMH locally decreases the sensitivity of FSH in follicles in multiple
462 species including the mouse and sheep (Durlinger *et al.* 2001; Campbell *et al.* 2012;
463 Visser and Themmen 2014). Recently, Ilha *et al.* (2016) reported that AMH mRNA levels
464 decrease in both dominant and subordinate follicles during follicular deviation in cows.
465 Thus, both dominant and subordinate follicles become more sensitive to FSH and can be
466 recruited to enter the pool of follicles which may then become dominant (Visser and
467 Themmen 2014). Therefore, AMH may have an important role in both the ovary and
468 gonadotrophs during follicular selection in monovulatory species.

469 Gonadotrophs are a heterogeneous cell population comprising LH and FSH
470 monohormonal and bihormonal subsets in rats, equines, and bovines (Townsend *et al.*
471 2004; Pals *et al.* 2008; Kadokawa *et al.* 2014). The fluorescent immunohistochemistry
472 showed the AMHR2 expression in LHβ-positive cells as well as FSHβ-positive cells. In
473 this study, 100 pg/ml and 1000 pg/ml of AMH stimulated LH secretion weakly. Therefore,

AMH may control also LH secretion, but weakly. Intraperitoneal injection with AMH increases FSH concentration in blood collected 18 h later in rats; however, AMH injection does not significantly increase LH concentration in the same blood samples (Garrel *et al*. 2016). Therefore, the effect of AMH on LH secretion *in vivo* may not become significant.

It is well known that GPCR proteins can form functionally active homomers and heteromers with different receptors (Ritter and Hall 2009). We obtained the strong positive overlap coefficient between AMHR2 and GnRHR on the cell-surface. This overlap coefficient was greater than that reported between GnRHR and flotillin-1 in cultured LβT2 cells (0.50; Wehmeyer *et al.* 2014) and similar to that we previously found between GnRHR and GPR61 (0.71; Pandey *et al.* 2017a) and GPR153 (0.75; Pandey *et al.* 2017b) in bovine gonadotrophs. Heterodimerization among paralogs of GnRHRs of a protochordate results in the modulation of ligand-binding affinity, signal transduction, and internalization (Satake *et al.* 2013). Thus, it is possible that AMHR2 forms a heteromer, affecting ligand-binding affinity, signal transduction, and internalization of GnRHR, and thus the synthesis and secretion of LH and FSH in AP of vertebrates. Furthermore, a recent study (Hossain *et al.* 2016) suggested that GPR61 form heteromers with other GPCRs. Therefore, further studies are required to clarify whether GnRHR form heteromers with GPR61, GPR153, and AMHR2.

In this study, we observed multiple, not single, bands of AMHR2 in western blotting, which has been reported previously. For example, Faure *et al.* (1996) reported three bands (82, 73, and 63 kDa) of dimers, full-length monomers, and cleaved monomers. Hirschhorn *et al.* (2015) reported more bands (~58 kDa, ~69 kDa, and ~71 kDa) of dimers, full-length monomers, and cleaved monomers. AMHR2 is present as dimers, full-length monomers, and cleaved monomers in bovine ovaries and APs. Treatment with *N*-glycosidase F shows a further two bands (68 kDa and 61 kDa) by cutting down by approximately 5 and 2 kDa, because AMHR2 is *O*-glycosylated (Faure *et al.* 1996). The full-length monomers in APs appeared as a doublet, whereas those in the ovary appeared as a single band in this study. Therefore, this study suggests that bovine AMHR2 is glycosylated, and the difference in the number of full-length monomers between the AP and ovary might be because of the glycosylation differences.

The anti-AMHR2 antibody revealed similar bands in the two tissues in the western blot. However, AP tissue showed weaker bands than ovarian tissue did. Nevertheless, β-actin bands showed weaker staining in both tissue types. This suggests that the AP cell lanes were loaded with a lower amount of proteins than expected. A second difference between AP and ovarian cells was the absence of the dimeric AMHR2 band in AP cells. However, this might be the consequence of the lower protein amount used in the AP cell

western blot. In fact, the high molecular weight band was detectable in the ovarian tissue extract only at the highest dose (i.e., 16 µg/lane).

We found that approximately 20% of AMHR2-positive cells were non-gonadotrophs. At the time of our manuscript preparation, no reports published on AMHR2 in non-gonadotrophs. An AMHR2 polymorphism (482 A>G) was associated with lower prolactin levels in women with polycystic ovary syndrome **(**Georgopoulos *et al.* 2013). Therefore, lactotrophs may express AMHR2 to play an important role in polycystic ovary syndrome, which is a possibility that bears further consideration in future investigations.

In conclusion, AMHR2 is expressed in the gonadotrophs of post-pubertal heifers to control gonadotropin secretion.

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Conflicts of Interest

The authors declare no conflicts of interest.

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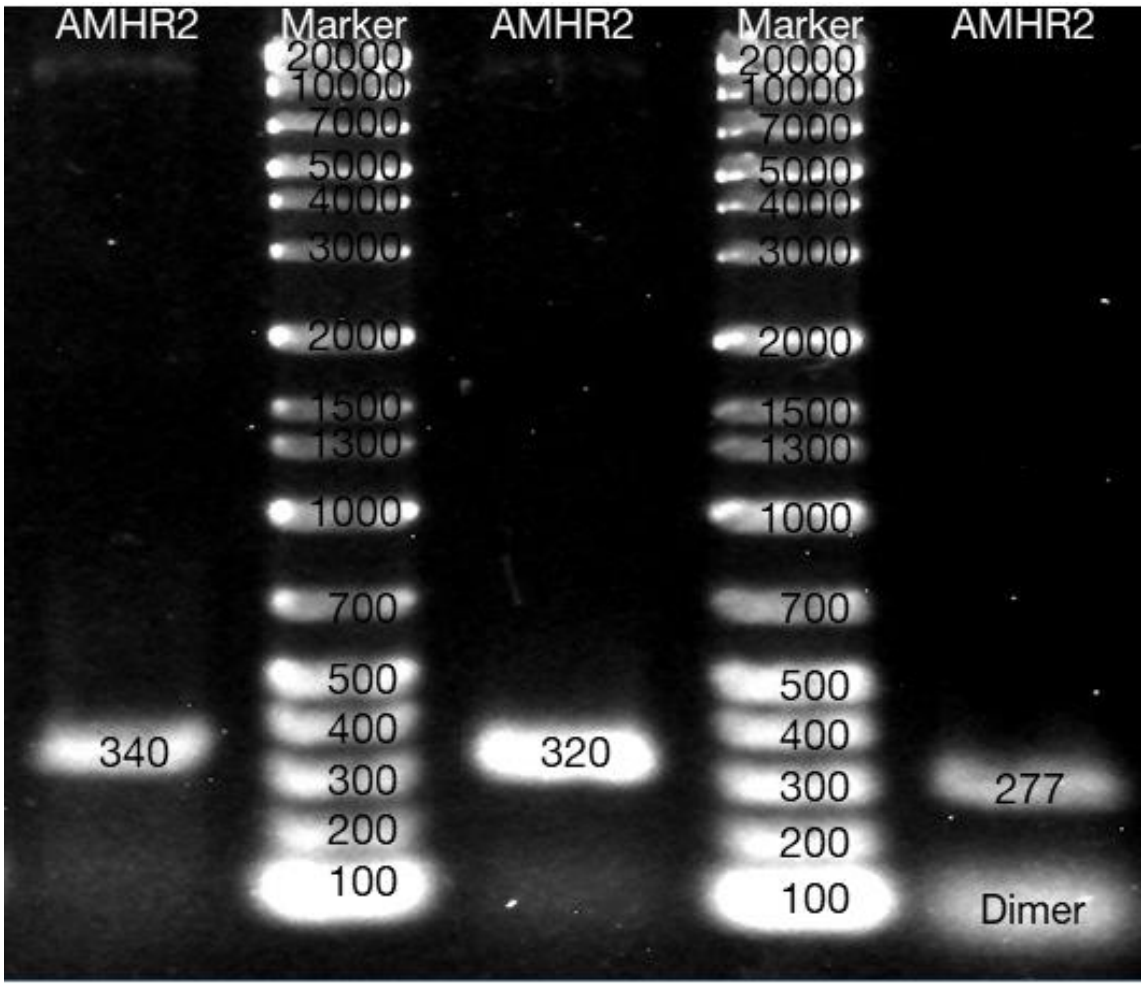
739 **Table 1.** Details of the three primers used for PCR to detect AMHR2 mRNA in bovine
740 anterior pituitaries.
741

Primer pair	Sequence	5'-3'	Position		Size (bp)
			Nucleotide	Exon	
1st	up	GATTTGCGACCTGACAGCAG	1273-1292	9-10	340
	down	CGGGAGGAGTGGAGAAATGG	1593-1612	11	
2nd	up	AGATTTGCGACCTGACAGCAG	1272-1292	9-10	320
	down	CTTCCAGGCAGCAAAGTGAG	1572-1591	11	
3rd	up	GTGCTTCTCCCAGGTCATACG	606-626	5-6	277
	down	GGTGTGCTGGGTCAAGTAGT	863-882	7	

742
743

744 **Figure Legends**



745

746 **Fig. 1.** Expression of anti-Müllerian hormone (AMH) receptor type 2 (AMHR2) mRNA
747 detected by RT-PCR. Electrophoresis of PCR-amplified DNA products using 1 of 3 pairs
748 of primers for bovine AMHR2 and cDNA derived from anterior pituitary (AP) of post-
749 pubertal heifers. The lanes labeled as AMHR2 demonstrate that the DNA products
750 obtained were of the size that had been expected—340 bp, 320bp, and 277 bp,
751 respectively. Other two lanes (Marker) are the DNA marker.

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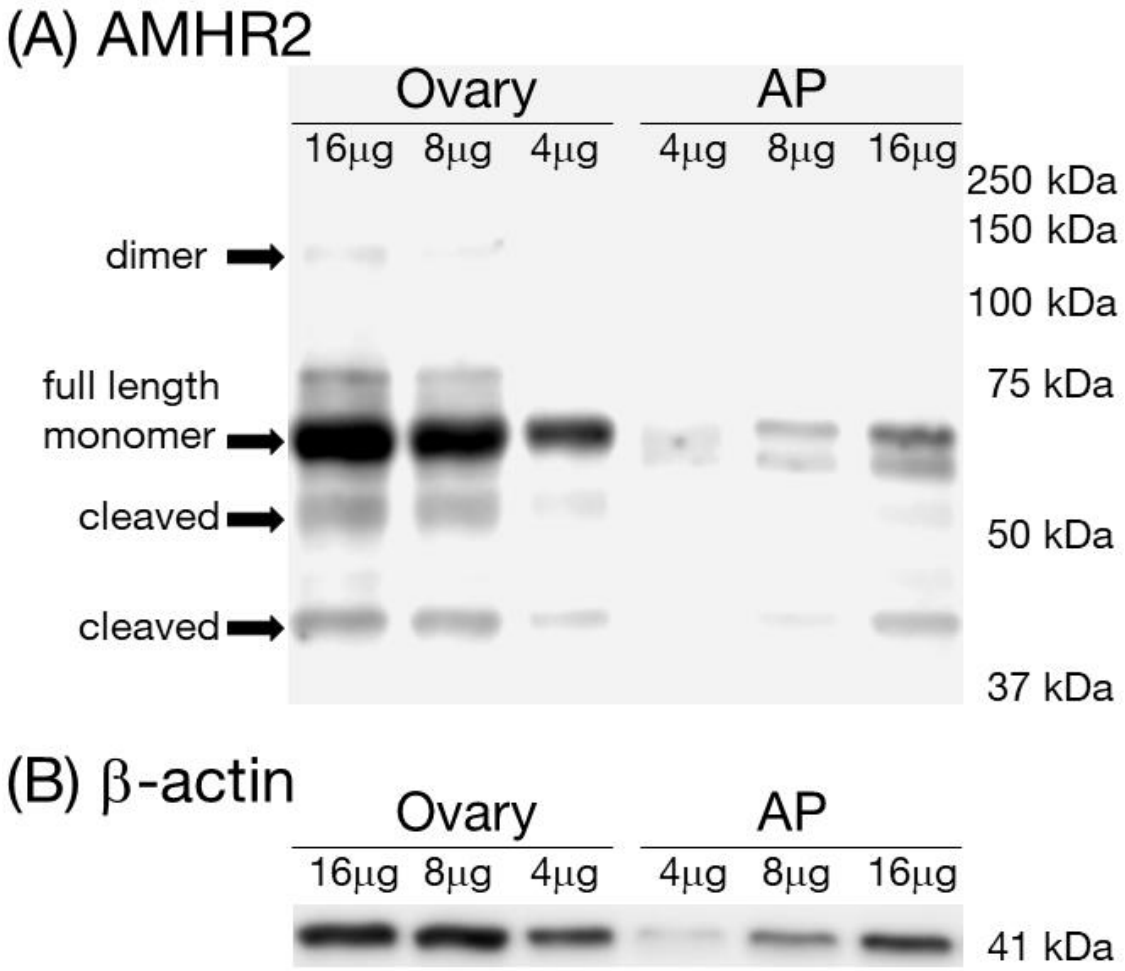

753

754

755 **Fig. 2.** Results of western blotting using extracts (4, 8, or 16 μg of total protein) from the

756 AP or ovary of post-pubertal heifers and anti-AMHR2 antibody (A) or anti-β–actin

757 antibody (B). We defined bovine AMHR2 bands based on size as dimers, full length

758 monomers, or cleaved monomers, according to previous studies utilizing western blotting

759 for human and mouse AMHR2 (Faure et al. 1996; Hirschhorn et al. 2015).

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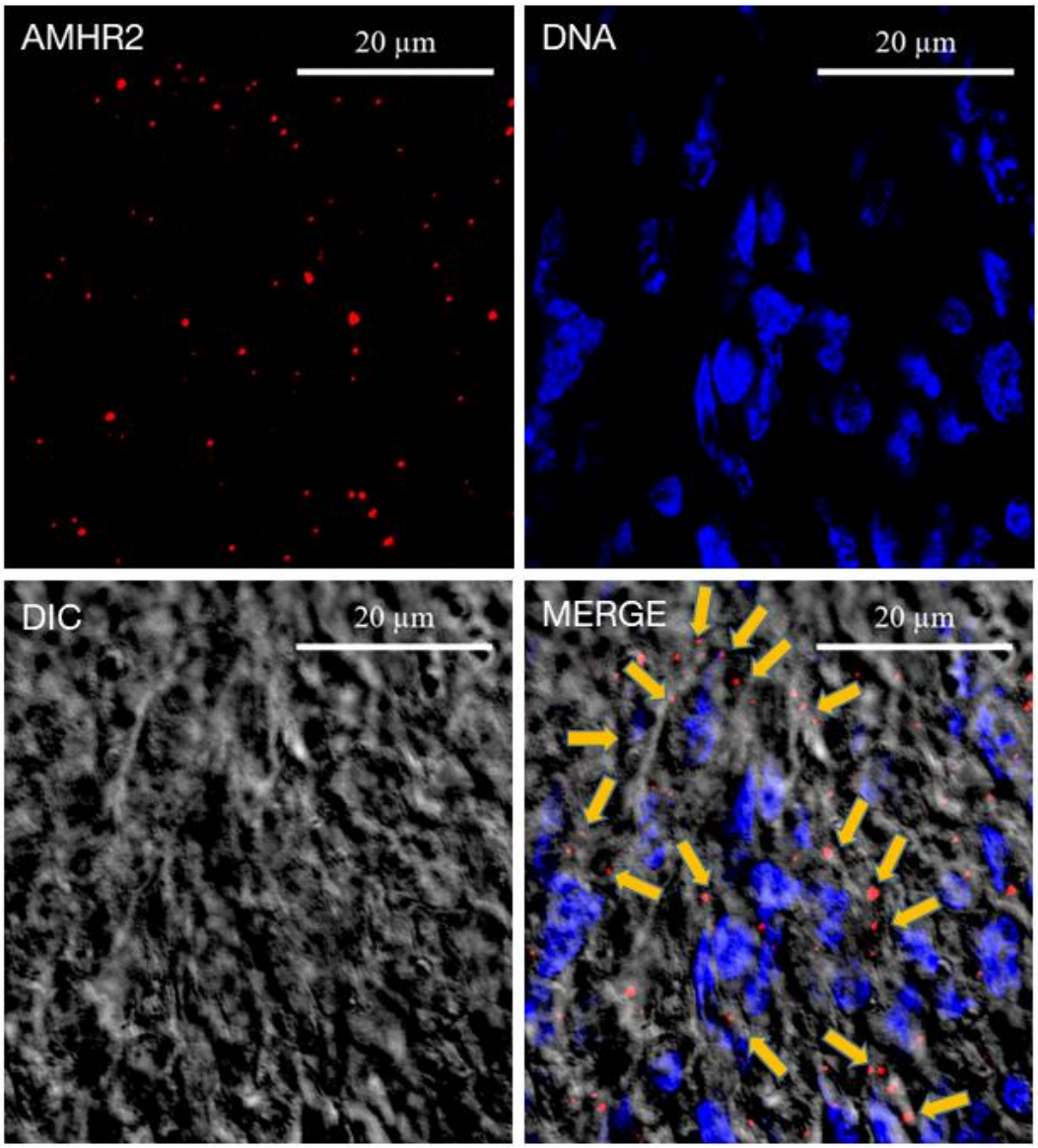


762

763 **Fig. 3.** Fluorescence immunocytochemistry was used to confirm the expression of

764 AMHR2 on the surface of granulosa cells of small (approximately 5 mm) follicles in the

765 ovaries of post-pubertal heifers. Images were captured by laser confocal microscopy for

766 AMHR2 (red), DNA (dark blue), and differential interference contrast (indicated as DIC).

767 Strong AMHR2 staining appeared to be aggregated (orange arrows), not evenly dispersed.

768 (scale bars = 20 µm)

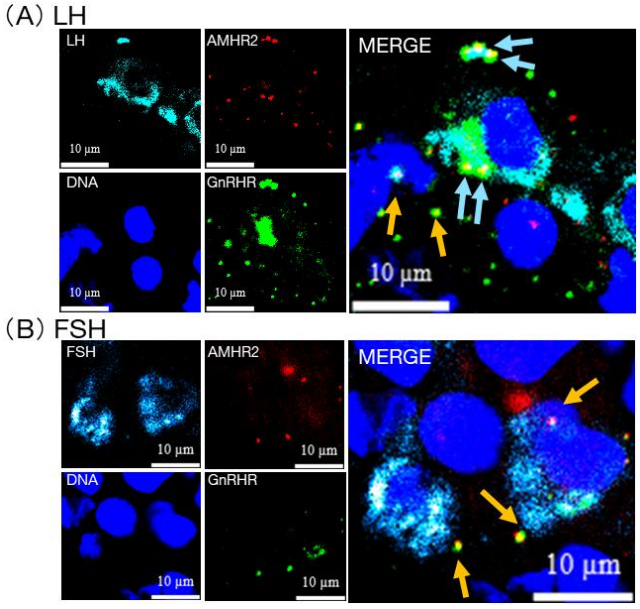


Fig. 4. Triple-fluorescence immunohistochemistry of AP tissue of post-pubertal heifers for AMHR2, gonadotropin-releasing hormone receptor (GnRHR) and either luteinizing hormone (LH) (A) or follicle stimulating hormone (FSH) (B). Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (green) and LH or FSH (light blue) with counter-staining by DAPI (dark blue). Yellow indicates the colocalization of AMHR2 and GnRHR on the surface of LH-positive cells (blue arrow) and FSH-positive cells (orange arrows). Both AMHR2 and GnRHR appeared to be aggregated, not evenly dispersed. Note that the focus depth of the high magnification lens is thin; thus, the best focus for the membrane receptors was quite different from both the best focus for the nucleus and the best focus for cytoplasmic LH. Therefore, this image was taken using the best focus for the membrane receptors while using strong laser power and strong CCD sensitivity for DAPI and cytoplasmic LH. Scale bars are 10 μm.

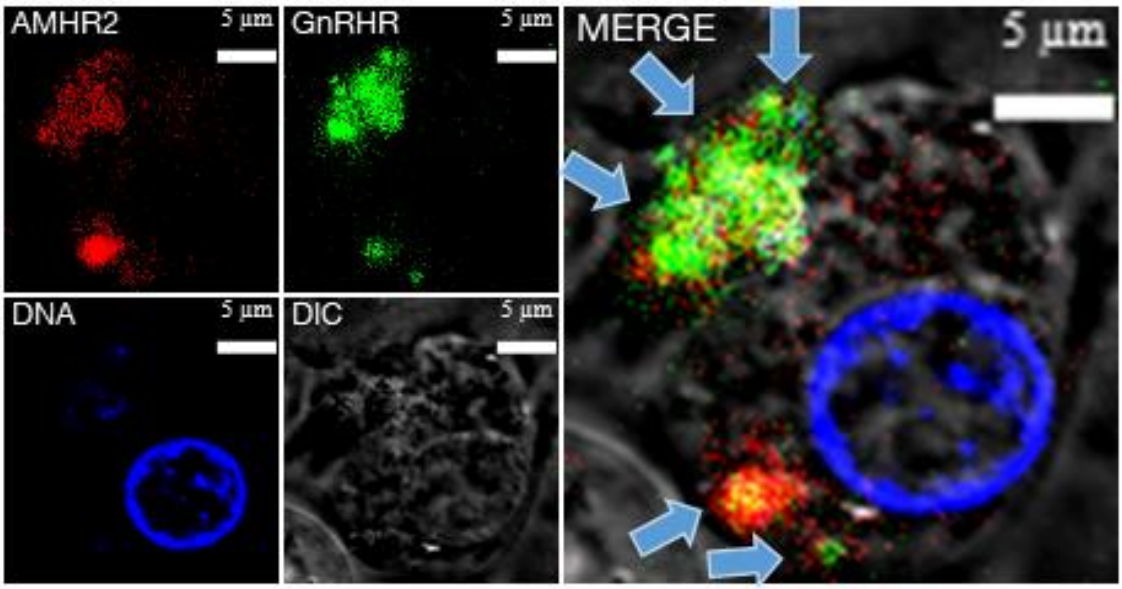
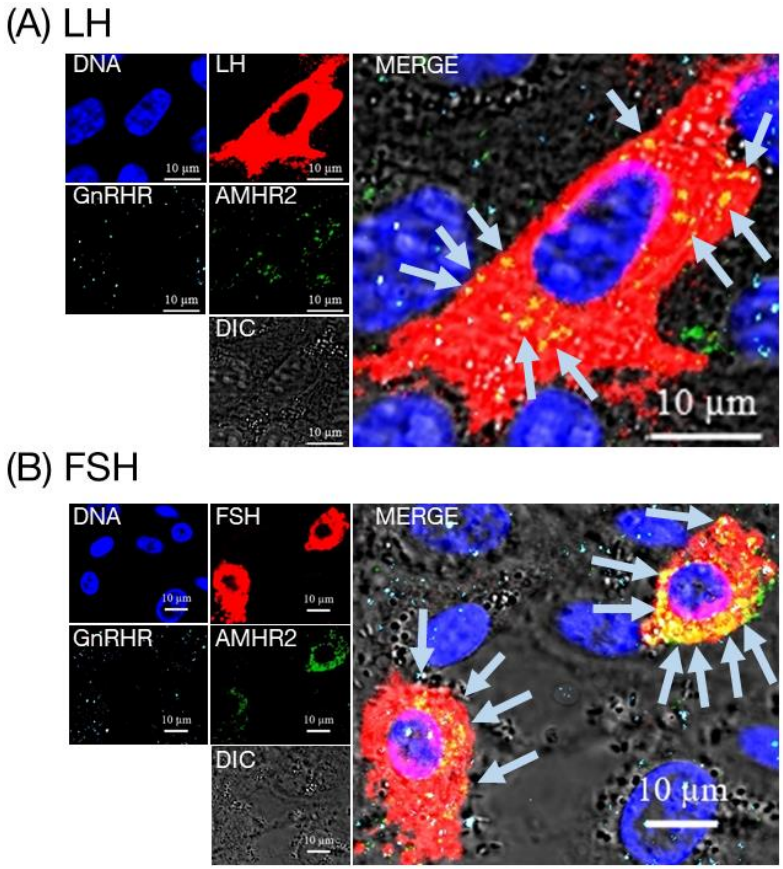


Fig. 5. Fluorescence immunocytochemistry was used to confirm the colocalization (yellow in the merge panel) of AMHR2 and GnRHR on the surface of cultured AP cells (prepared by CellCover method) of post-pubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (green), DNA (dark blue), and DIC on cultured AP cells which did not receive Triton X-100 treatment for antibody penetration. Thus, antibody could only bind AMHR2 and GnRHR on the surface of gonadotrophs. The blue arrows indicate the colocalization of aggregated GnRHR and aggregated AMHR2. (scale bars = 5 µm).



797

798 **Fig. 6.** Triple-fluorescence immunocytochemistry of cultured AP cells (prepared by PFA-
799 Triton method) of post-pubertal heifers for AMHR2, GnRHR and either LH (A) or FSH
800 (B). Images were captured by laser confocal microscopy for AMHR2 (green), GnRHR
801 (light blue) and LH or FSH (red) with counter-staining by DAPI (dark blue). Yellow
802 (shown by arrows) indicates the colocalization of AMHR2 and LH of FSH in LH-positive
803 cells (A) and FSH-positive cells (B). This image was taken using the best focus for the
804 membrane receptors while using strong laser power and strong CCD sensitivity for DAPI
805 and cytoplasmic LH. Note that the cells prepared by the PFA-triton method are thinner
806 than those prepared by the CellCover method. Scale bars are 10 µm.

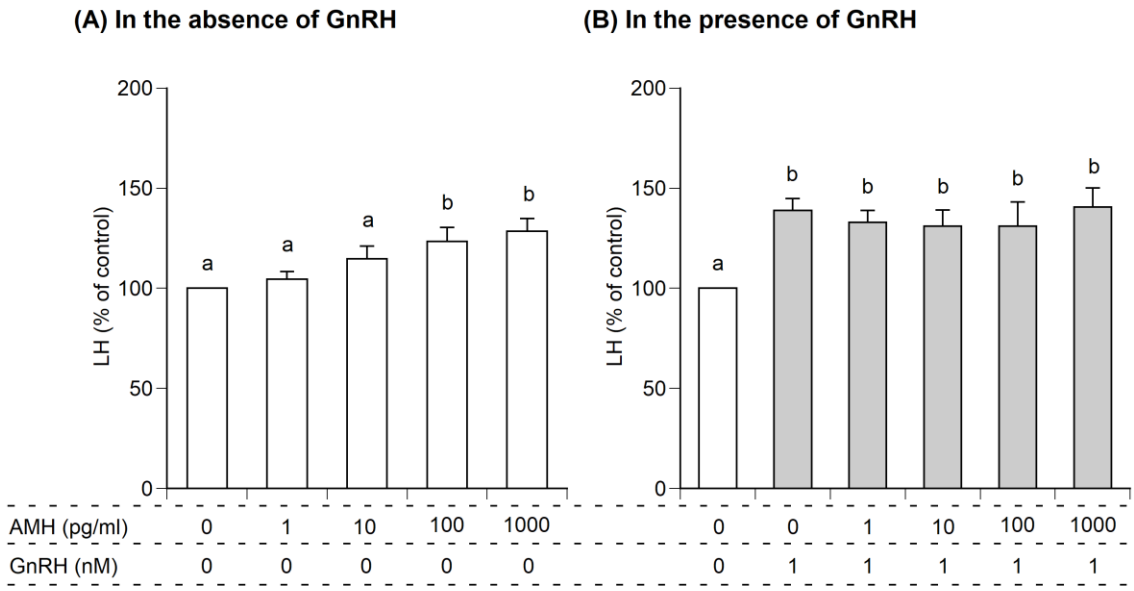


Fig. 7. Comparison of the effects of various concentrations of AMH in media with (A) and without (B) 1 nM GnRH on LH secretion from cultured AP cells of post-pubertal heifers. The concentrations of LH in the control cells (cultured in medium alone without AMH and GnRH) were averaged and set at 100%, and the mean LH concentration for each treatment group is expressed as a percentage of the control value. Different letters indicate statistical differences ($P < 0.05$).

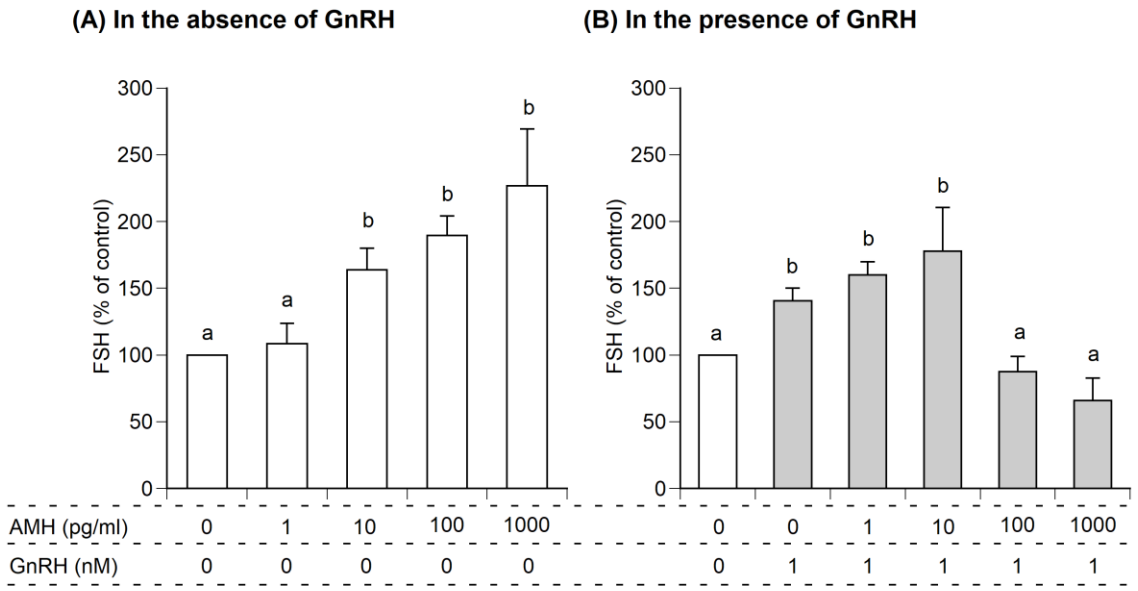


Fig. 8. Comparison of the effects of various concentrations of AMH in media with (A) and without (B) 1 nM GnRH on FSH secretion from cultured AP cells of post-pubertal heifers. The concentrations of FSH in the control cells (cultured in medium alone without AMH and GnRH) were averaged and set at 100%, and the mean FSH concentration for each treatment group is expressed as a percentage of the control value. Different letters indicate statistical differences ($P < 0.05$).