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.

Short summary

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

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) at the surface. GnRHRs are present in gonadotroph plasma membrane lipid rafts (Navratil *et al.* 2009; Wehmeyer *et al.* 2014; Kadokawa *et al.* 2014), which are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons *et al.* 2000; Head *et al.* 2014). Lipid rafts 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

frozen in liquid nitrogen and stored at −80°C. The AP and ovary samples for immunohistochemistry (n = ~~3~~5) were fixed with 4% paraformaldehyde at 4°C for 16 h. The AP samples meant for cell culture followed by immunocytochemical analysis (n = 5) and those that were to be used for cell culture to evaluate the effect of AMH on LH and FSH secretion (n = 8) were stored in ice-cold 25 mM HEPES buffer (pH 7.2) containing 10 mM glucose and transported on ice to the laboratory.

RT-PCR, sequencing of amplified products, and homology search in gene databases

Total RNA was extracted from the AP samples (n = 3) using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The extracted RNA samples were treated with ribonuclease-free deoxyribonuclease (Toyobo, Tokyo, Japan) to eliminate possible genomic DNA contamination. The concentration and purity of each RNA sample were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) to ensure the A_{260}/A_{280} nm ratio was in the acceptable range of 1.8–2.1. Electrophoresis of total RNA followed by staining with ethidium bromide was performed to verify the mRNA quality of all samples, and 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 obtained sequences 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 confirmed 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 to synthesize antigen peptide (C-GVRGSTQNLGKLLDA), conjugation with keyhole limpet hemocyanin (KLH), immunization, and antibody purification. Briefly, the AMHR2 antigen peptide was synthesized, and the purity was verified (greater than 99.0%) using high-performance liquid chromatography followed by mass spectrometry. Then, KLH was conjugated to the sulfhydryl group of the cysteine of the antigen peptide. The immunogen was emulsified with Complete Freund's adjuvant and injected to chickens five times at 14-day intervals. Blood was collected 7 days after the final immunization. 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-

168 terminal extracellular domain (corresponding to amino acids 1–29;
169 MANSDSPEQNENHCSAINSSIPLTPGSLP) of GnRHR (anti-GnRHR). The specificity
170 of the anti-GnRHR antibody was verified by western blotting, and pretreatment with anti-
171 GnRHR antibody inhibited GnRH-induced LH secretion from cultured bovine
172 gonadotroph (Kadokawa *et al*. 2014). Additionally, we previously used the anti-GnRHR
173 antibody for immunofluorescence detection of GnRHR in plasma membrane of bovine
174 gonadotroph (Kadokawa *et al*. 2014; Pandey *et al.* 2016). We observed a strong and
175 localized GnRHR-positive staining signal as aggregation on the plasma membrane of
176 gonadotrophs (Kadokawa *et al.* 2014). We used the anti-GnRHR as well as a mouse
177 monoclonal anti-LH β (LHβ) subunit antibody (clone 518-B7; Matteri *et al.* 1987) for
178 immunohistochemical analysis of AP tissue and cultured AP cells. This antibody does not
179 cross-react with other pituitary hormones (Iqbal *et al.* 2009). Also we used a mouse
180 monoclonal anti-FSH β (FSHβ) subunit antibody (clone A3C12) that does not cross-react
181 with other pituitary hormones (Borromeo *et al.* 2004) for immunohistochemical analysis
182 of AP tissue.
183
184 *Western Blotting for AMHR2*
185 Briefly, we extracted protein from the AP (n = 3) or ovary (n = 3, used as positive

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

204 monomers, or cleaved monomers (Faure *et al.* 1996; Hirschhorn *et al.* 2015). Thus, we
205 defined bovine AMHR2 bands based on mobility as one of these structure types. After
206 antibodies were removed from the PVDF membrane with stripping solution (Nacalai
207 Tesque Inc., Kyoto, Japan), the membrane was used for immunoblotting with the anti-β-
208 actin mouse monoclonal antibody (A2228, 1:50,000 dilution; Sigma-Aldrich, St. Louis,
209 MO, USA).
210
211 *Fluorescent immunohistochemistry and confocal microscopic observation*
212 After storage in 4% paraformaldehyde PBS at 4°C for 16 h, the AP (n = ~~3~~ 5) or ovary
213 (n = 5) tissue blocks were placed in 30% sucrose PBS until the blocks were infiltrated
214 with sucrose. The methods for immunofluorescence analysis of AP tissue have been
215 described previously (Kadokawa *et al.* 2014). Briefly, we prepared 15-μm sagittal
216 sections and mounted them on slides. The sections were treated with 0.3 % Triton X-100
217 in PBS for 15 min, then, incubated with 0.5 mL of PBS containing 10% normal goat
218 serum (Wako Pure Chemicals, Osaka, Japan) for blocking for 1 h. Incubation with a
219 cocktail of primary antibodies (anti-GnRHR guinea pig antibody, anti-AMHR2 chicken
220 antibody, and either anti-LHβ or anti-FSHβ mouse antibody [all diluted as 1:1,000]) for
221 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 by confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany) equipped with a diode laser 405 nm, argon laser 488 nm, HeNe laser 533 nm, and HeNe laser 633 nm. 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 percentage of GnRHR single (green)-labeled light blue-colored cells, or the percentage of double (yellow)-labeled light blue-colored cells, among all of the GnRHR-positive light blue-colored cells (sum of the numbers of green-labeled and yellow-labeled light blue-colored cells), were determined from 12 representative confocal images per pituitary gland. To verify the specificity of the signals, we included several negative controls in which the primary antiserum had been omitted or pre-absorbed with 5 nM of the same antigen peptide, or in which normal chicken IgG (Wako Pure Chemicals) was used instead of the primary antibody.

AP cell culture and immunocytochemical analysis of cells

Enzymatic dispersal of the AP cells from 5 heifers was performed using a previously described method (Suzuki *et al.* 2008) and confirmation of cell viability of greater than 90% was determined via Trypan blue exclusion. Total cell yield was $19.8 \times 10^6 \pm 0.8 \times 10^6$ cells per pituitary gland. The dispersed cells were then suspended in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific) containing 1×

258 nonessential amino acids (Thermo Fisher Scientific), 100 U/mL penicillin, 50 μg/mL
259 streptomycin, 10% horse serum (Thermo Fisher Scientific), and 2.5% fetal bovine serum
260 (Thermo Fisher Scientific). The cells (2.5×10^5 cells/mL, total = 0.15 mL per lane) were
261 cultured in the culture medium at 37 °C in 5% CO_2 for 82 h, using a microscopy chamber
262 (μ-Slide VI 0.4, Ibidi, Planegg, Germany). We cultured the AP cells for 82 h (3.5 days),
263 as previously described (Hashizume *et al.* 2003; Kadokawa *et al.* 2008; Hashizume *et al.*
264 2009; Kadokawa *et al.* 2014; Nakamura *et al.* 2015). We supplied recombinant human
265 activin A (final concentration, 10 ng/ml; R&D systems, Minneapolis, MN, US) to
266 stimulate FSH synthesis at 24 h prior to fixation. Mature activin A of bovines
267 (NP_776788.1) and ovines (NP_001009458.1) have 100% homology with that of humans
268 (CAA40805.1), and the 24 h culture with the same concentration of same recombinant
269 human activin A product stimulates FSH expression in cultured ovine AP cells (Young *et*
270 *al.* 2008).

271 We fixed and treated the cultured cells by using either (1) 4% paraformaldehyde
272 fixation for 3 min and 0.1% Triton X-100 treatment for 1 min (PFA-Triton method) or (2)
273 CellCover (Anacyte aboratories UG, Kuhreder, Hamburg) for fixation, instead of 4% PFA,
274 for 2 min, and no Triton X-100 treatment (CellCover method). These methods were
275 described previously (Kadokawa *et al.* 2014). Briefly, cells attached to the bottom of the

276 microscopy chamber were treated following one of the aforementioned methods. For the
277 PFA-Triton method, the fixed cells were incubated with 0.1 mL of the same cocktail of
278 primary antibodies for 2 h at room temperature. Incubation with Triton X-100 allowed
279 both anti-GnRHR and anti-AMHR2 antibodies to bind to target proteins in the cytoplasm
280 and at the cell surface. For the CellCover method, the fixed cells were incubated with
281 only guinea pig anti-GnRHR and chicken anti-AMHR2 antibodies (both 1:1,000) for 2 h
282 at room temperature. Since the cells were not treated with Triton X-100, the antibodies
283 bound only to the extracellular domains of the respective receptors in most cells, although
284 some cytoplasmic labeling occurred in broken cells. For both PFA-Triton and CellCover
285 methods, cells were incubated with fluorochrome-conjugated secondary antibody
286 cocktail and DAPI. The cells were visualized by confocal microscopy and fluorescence
287 micrographs and differential interference contrast (DIC) images were obtained on a single
288 plane. Signal specificity was confirmed using negative controls in which the primary
289 antiserum was omitted or pre-absorbed with 5 nM antigen peptide, or in which the normal
290 chicken IgG replaced the primary antibody. Eight randomly selected images of cells
291 prepared by CellCover method were analyzed for co-localization utilizing the ZEN 2012
292 black edition software (Carl Zeiss) to calculate overlap coefficients (Manders *et al.* 1993)
293 for the Alexa Fluor 488 and Alexa Fluor 647 fluorophores.

294

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

296 The AP cells derived from 8 heifers were prepared using the protocol described above.
297 After the cells (2.5×10^5 cells/mL, total 0.3 mL) had been plated in 48-well culture plates
298 (Sumitomo Bakelite, Tokyo, Japan), they were maintained at 37ºC in a humidified
299 atmosphere of 5% CO_2 for 82 h. We supplied the recombinant human activin A (final
300 concentration, 10 ng/ml) to stimulate FSH synthesis at 24 h prior to the AMH test.

301 In the test to evaluate the effect of AMH in the absence of GnRH, the old medium
302 was replaced by 295 μL DMEM containing 0.1% BSA and 10 ng/ml activin A and
303 incubated for 2 h. Treatment was performed by adding 5 μL of DMEM alone or 5 μL of
304 DMEM containing various concentrations of human recombinant AMH (R & D systems;
305 final concentration of 0, 1, 10, 100, or 1000 pg/ml AMH).

306 The bioactive region in the carboxyl-terminal region of mature AMH (Belville *et al.*
307 2004) of bovines (NP_776315.1) and goat (XP_017906255.1) has 96% homology with
308 that of humans (NP_000470.2), and the same recombinant human AMH product shows
309 the biological effect for goat follicles (Rocha *et al.* 2016).

310 After incubation for further 2 h, the medium from each well was collected for
311 radioimmunoassay (RIA) analyses of LH and FSH levels. The physiological

312 concentration of AMH in blood ranged between 5 and 300 pg/ml in Japanese Black cows
313 in our previous study (Koizumi and Kadokawa 2017). Therefore, we used the above-
314 mentioned AMH concentration in this study.

315 In the test to evaluate the effect of AMH in the presence of GnRH, the old medium
316 was replaced by 290 μL DMEM containing 0.1% BSA and 10 ng/ml activin A and
317 incubated at 37ºC for 2 h. Pretreatment was performed by adding 5 μL of DMEM alone
318 or 5 μL of DMEM containing various concentrations (0, 60, 600, 6000, and 60000 pg/ml)
319 of the human recombinant AMH. The cells were incubated while gently shaking for 5
320 min, and then, cells were treated with 5 μL of 60 nM GnRH (Peptide Institute Inc., Osaka,
321 Japan) dissolved in DMEM for 2 h in order to stimulate LH and FSH secretion. The
322 pretreatment plus the GnRH treatment yielded a final concentration of 0, 1, 10, 100, or
323 1000 pg/ml AMH. The final concentration of GnRH was 1 nM in all treatments
324 (Kadokawa *et al.* 2014), except the "control". Control wells were treated with 5 μL of
325 DMEM, but were not incubated with GnRH. "GnRH" wells were pre-treated with 5 μL
326 of DMEM for 5 min and were then incubated with GnRH for 2 h. After incubation for 2
327 h, the medium from each well was collected for LH and FSH RIAs.

328

329 *RIAs to measure gonadotropin concentration in culture media*

LH concentrations in the culture media were assayed in duplicate by double antibody RIA using ^{125}I-labeled bLH and anti-oLH-antiserum (AFP11743B and AFP192279, National Hormone and Pituitary Program of the National Institute of 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- and inter-assay coefficients of variation were 3.6% and 6.2%, respectively. FSH concentrations in the culture media were assayed in duplicate 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- and inter-assay coefficients of variation were 4.3% and 7.1%, respectively.

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

366 that the full-length monomer in the ovary appeared as a single band, whereas in AP cells,
367 it appeared as a doublet (Fig. 2A). No bands were observed in the negative control
368 membranes, where the primary antiserum was pre-absorbed with the antigen peptide.
369
370 *Immunofluorescence analysis of AMHR2 expression in bovine granulosa cells*
371 Fig. 3 shows the immunofluorescence in the granulosa cells of small (about 5 mm)
372 follicles in the ovary tissues of post-pubertal heifers. Strong AMHR2 staining appeared
373 to be aggregated, not evenly dispersed.
374
375 *Immunofluorescence analysis of AMHR2 expression in bovine AP tissue*
376 Expression of LHβ, FSHβ, GnRHR, and AMHR2 in bovine AP tissue was
377 investigated by immunohistochemistry (Fig. 4). AMHR2 and GnRHR colocalized in the
378 majority of both LHβ-positive (Fig. 4A) and FSHβ-positive (Fig. 4B) cells. Focus depth
379 of the high magnification lens used in this study are thin, thus, the best focus for GnRHR
380 and AMHR2 on plasma membrane was quite different from both the best focus for
381 nucleus and the best focus for cytoplasmic LHβ or FSHβ. Thus, we could know both
382 membrane receptors are on the cell-surface. Percentages of single- and double-labeled
383 AMHR2- and GnRHR-positive cells were determined from 12 representative confocal

384 images per pituitary gland. In each pituitary gland, there was an average of 52.4 ± 2.4
385 GnRHR-positive cells, 44.6 ± 1.2 AMHR2-positive cells, and 33.6 ± 1.3 double-positive
386 cells; 64.5% ± 3.2% of GnRHR-positive cells were AMHR2-positive, whereas 78.4% ±
387 1.8% of AMHR2-positive cells were GnRHR-positive.
388
389 *AMHR2 and GnRHR aggregate on the surface of cultured AP cells*
390 In the AP cells prepared by the CellCover method, AMHR2 aggregated on the surface
391 of GnRHR-positive cells (Fig. 5). The overlap coefficient between AMHR2 and GnRHR
392 was 0.76 ± 0.05 on the cell surface of cultured AP cells.
393
394 *AMHR2 expression in cultured gonadotrophs*
395 Among the AP cells prepared by the PFA-Triton method, we observed AMHR2 in both
396 LHβ-positive and FSHβ-positive cells (Fig. 6).
397
398 *Effects of AMH on gonadotropin secretion from cultured AP cells*
399 Fig. 7 shows the effect of various concentrations of AMH on LH secretion from the
400 AP cells derived from post-pubertal heifers cultured in the absence (A) or presence (B)
401 of GnRH. In the absence of GnRH (Fig. 7A), 100 pg/ml and 1000 pg/ml of AMH

increased ($P < 0.05$) LH secretion, when compared with the controls (17.6 ± 2.4 ng/ml). Conversely, there was no effect of AMH on the GnRH-induced LH secretion (Fig. 7B).

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

420 strong signal located on the surface of granulosa cells in small antral follicles, where
421 AMHR2 mRNA is expressed (Poole *et al.* 2016). Therefore, the anti-bovine AMHR2 is
422 the first developed tool that can be used for immunohistochemistry in bovine samples.

423 In this study, treatment with 10–1000 pg/ml of AMH stimulated FSH secretion in the
424 absence of GnRH. This agrees with *in vivo* experiments on rats, where AMH stimulates
425 the secretion and expression of FSH (Garrel *et al.* 2016). These data suggested that AMH
426 might bind with AMHR2 to increase FSH secretion from gonadotroph in ruminants as
427 well. Garrel *et al*. (2016) recently reported that AMH increases both FSHβ expression
428 and phosphorylates SMAD 1/5/8 in LβT2 cells, but such increases are blocked by GnRH.
429 In this study, 1–10 pg/ml AMH did not change GnRH-stimulated FSH secretion; however,
430 100–1000 pg/ml AMH suppressed GnRH-stimulated FSH secretion. Therefore, further
431 studies are required to clarify the molecular mechanisms controlling FSH secretion from
432 ruminant gonadotrophs by AMH and GnRH, especially whether the SMAD 1/5/8
433 pathways have important roles.

434 Multiparous (third parity or higher) Japanese Black cows have significantly higher
435 blood AMH concentrations (100 pg/ml level) than primiparous cows (1–10 pg/ml level)
436 throughout the postpartum period (Koizumi and Kadokawa 2017). The multiparous
437 Japanese Black cows have larger number of days from parturition to postpartum first

438 ovulation than the primiparous cows (Koizumi and Kadokawa 2016). Therefore, the
439 suppressing effect of 100–1000 pg/ml of AMH on GnRH-stimulated FSH secretion may
440 have an important role in the follicular growth and delayed postpartum first ovulation in
441 multiparous cows.

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

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

456 Koizumi and Kadokawa 2017). Therefore, we must be cautious when concluding that
457 AMH contributes largely in controlling LH and FSH secretion from gonadotrophs *in vivo*.
458 Our results suggested that preantral and small antral follicles may control
459 gonadotropin secretion from the AP in post-pubertal heifers. Conversely, FSH suppresses
460 AMH secretion from bovine granulosa cells (Rico *et al.* 2011). Therefore, there may be
461 feedback mechanisms between gonadotrophs and granulosa cells in preantral and small
462 antral follicles. AMH locally decreases the sensitivity of FSH in follicles in multiple
463 species including the mouse and sheep (Durlinger *et al.* 2001; Campbell *et al.* 2012;
464 Visser and Themmen 2014). Recently, Ilha *et al.* (2016) reported that AMH mRNA levels
465 decrease in both dominant and subordinate follicles during follicular deviation in cows.
466 Thus, both dominant and subordinate follicles become more sensitive to FSH and can be
467 recruited to enter the pool of follicles which may then become dominant (Visser and
468 Themmen 2014). Therefore, AMH may have an important role in both the ovary and
469 gonadotrophs during follicular selection in monovulatory species.
470 Gonadotrophs are a heterogeneous cell population comprising LH and FSH
471 monohormonal and bihormonal subsets in rats, equines, and bovines (Townsend *et al.*
472 2004; Pals *et al.* 2008; Kadokawa *et al.* 2014). The fluorescent immunohistochemistry
473 showed the AMHR2 expression in LHβ-positive cells as well as FSHβ-positive cells. In

474 this study, 100 pg/ml and 1000 pg/ml of AMH stimulated LH secretion weakly. Therefore,
475 AMH may control also LH secretion, but weakly. Intraperitoneal injection with AMH
476 increases FSH concentration in blood collected 18 h later in rats; however, AMH injection
477 does not significantly increase LH concentration in the same blood samples (Garrel *et al*.
478 2016). Therefore, the effect of AMH on LH secretion *in vivo* may not become significant.
479 It is well known that GPCR proteins can form functionally active homomers and
480 heteromers with different receptors (Ritter and Hall 2009). We obtained the strong
481 positive overlap coefficient between AMHR2 and GnRHR on the cell-surface. This
482 overlap coefficient was greater than that reported between GnRHR and flotillin-1 in
483 cultured LβT2 cells (0.50; Wehmeyer *et al.* 2014) and similar to that we previously found
484 between GnRHR and GPR61 (0.71; Pandey *et al.* 2017a) and GPR153 (0.75; Pandey *et*
485 *al.* 2017b) in bovine gonadotrophs. Heterodimerization among paralogs of GnRHRs of a
486 protochordate results in the modulation of ligand-binding affinity, signal transduction,
487 and internalization (Satake *et al.* 2013). Thus, it is possible that AMHR2 forms a
488 heteromer, affecting ligand-binding affinity, signal transduction, and internalization of
489 GnRHR, and thus the synthesis and secretion of LH and FSH in AP of vertebrates.
490 Furthermore, a recent study (Hossain *et al.* 2016) suggested that GPR61 form heteromers

491 with other GPCRs. Therefore, further studies are required to clarify whether GnRHR form
492 heteromers with GPR61, GPR153, and AMHR2.

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

505 The anti-AMHR2 antibody revealed similar bands in the two tissues in the western
506 blot. However, AP tissue showed weaker bands than ovarian tissue did. Nevertheless, β-
507 actin bands showed weaker staining in both tissue types. This suggests that the AP cell
508 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.

Acknowledgments

Both Onalenna Kereilwe and Kiran Pandey were supported by MEXT (Ministry of Education, Culture, Sports, Science, and Technology) with the provision of a scholarship. This research was partly supported by a Grant-in Aid for Scientific Research from Yamaguchi University Foundation (Yamaguchi, Japan) to Hiroya Kadokawa. The authors

thank Dr. A. F. Parlow of National Hormone & Peptide Program (Harbor-UCLA medical center Torrance, CA, U.S.A.) for supplying the RIA kits.

Conflicts of Interest

The authors declare no conflicts of interest.

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735 **Table 1.** Details of the three primers used for PCR to detect AMHR2 mRNA in bovine

736 anterior pituitaries.

737

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	

738

739

740 **Figure Legends**

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

747

748 **Fig. 2.** Results of western blotting using extracts (4, 8, or 16 μg of total protein) from the
749 AP or ovary of post-pubertal heifers and anti-AMHR2 antibody (A) or anti-β–actin
750 antibody (B). We defined bovine AMHR2 bands based on size as dimers, full length
751 monomers, or cleaved monomers, according to previous studies utilizing western blotting
752 for human and mouse AMHR2 (Faure et al. 1996; Hirschhorn et al. 2015).

753

754 **Fig. 3.** Fluorescence immunocytochemistry was used to confirm the expression of
755 AMHR2 on the surface of granulosa cells of small (approximately 5 mm) follicles in the
756 ovaries of post-pubertal heifers. Images were captured by laser confocal microscopy for
757 AMHR2 (red), DNA (dark blue), and differential interference contrast (indicated as DIC).

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

759 (scale bars = 20 μm)

760

761 **Fig. 4.** Triple-fluorescence immunohistochemistry of AP tissue of post-pubertal heifers

762 for AMHR2, gonadotropin-releasing hormone receptor (GnRHR) and either luteinizing

763 hormone (LH) (A) or follicle stimulating hormone (FSH) (B). Images were captured by

764 laser confocal microscopy for AMHR2 (red), GnRHR (green) and LH or FSH (light blue)

765 with counter-staining by DAPI (dark blue). Yellow indicates the colocalization of

766 AMHR2 and GnRHR on the surface of LH-positive cells (blue arrow) and FSH-positive

767 cells (orange arrows). Both AMHR2 and GnRHR appeared to be aggregated, not evenly

768 dispersed. Note that the focus depth of the high magnification lens is thin; thus, the best

769 focus for the membrane receptors was quite different from both the best focus for the

770 nucleus and the best focus for cytoplasmic LH. Therefore, this image was taken using the

771 best focus for the membrane receptors while using strong laser power and strong CCD

772 sensitivity for DAPI and cytoplasmic LH. Scale bars are 10 μm.

773

774 **Fig. 5.** Fluorescence immunocytochemistry was used to confirm the colocalization

775 (yellow in the merge panel) of AMHR2 and GnRHR on the surface of cultured AP cells

776 (prepared by CellCover method) of post-pubertal heifers. Images were captured by laser
777 confocal microscopy for AMHR2 (red), GnRHR (green), DNA (dark blue), and DIC on
778 cultured AP cells which did not receive Triton X-100 treatment for antibody penetration.
779 Thus, antibody could only bind AMHR2 and GnRHR on the surface of gonadotrophs.
780 The blue arrows indicate the colocalization of aggregated GnRHR and aggregated
781 AMHR2. (scale bars = 5 μm).
782
783 **Fig. 6.** Triple-fluorescence immunocytochemistry of cultured AP cells (prepared by PFA-
784 Triton method) of post-pubertal heifers for AMHR2, GnRHR and either LH (A) or FSH
785 (B). Images were captured by laser confocal microscopy for AMHR2 (green), GnRHR
786 (light blue) and LH or FSH (red) with counter-staining by DAPI (dark blue). Yellow
787 (shown by arrows) indicates the colocalization of AMHR2 and LH of FSH in LH-positive
788 cells (A) and FSH-positive cells (B). This image was taken using the best focus for the
789 membrane receptors while using strong laser power and strong CCD sensitivity for DAPI
790 and cytoplasmic LH. Note that the cells prepared by the PFA-triton method are thinner
791 than those prepared by the CellCover method. Scale bars are 10 μm.
792

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 ($P < 0.05$).

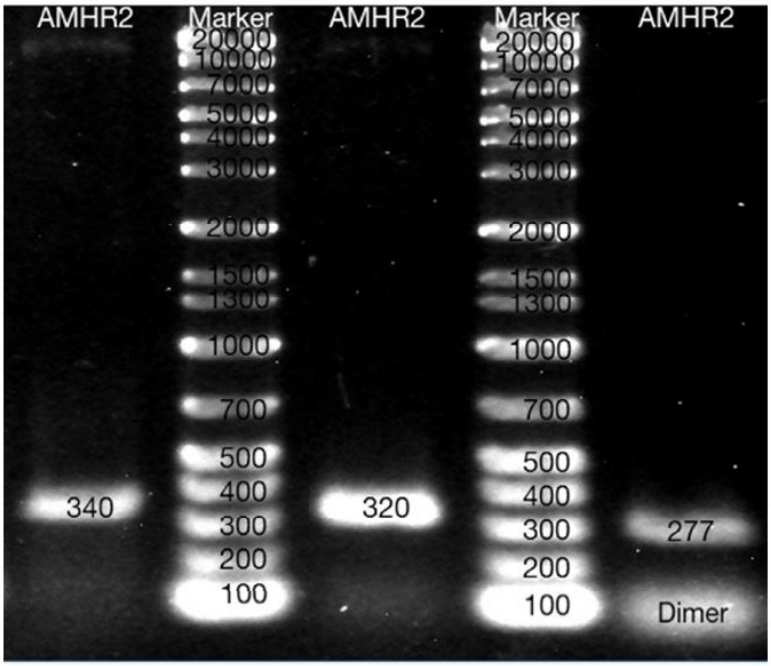

Fig. 1. Expression of anti-Müllerian hormone (AMH) receptor type 2 (AMHR2) mRNA detected by RT-PCR. Electrophoresis of PCR-amplified DNA products using 1 of 3 pairs of primers for bovine AMHR2 and cDNA derived from anterior pituitary (AP) of post-pubertal heifers. The lanes labeled as AMHR2 demonstrate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

44x38mm (300 x 300 DPI)

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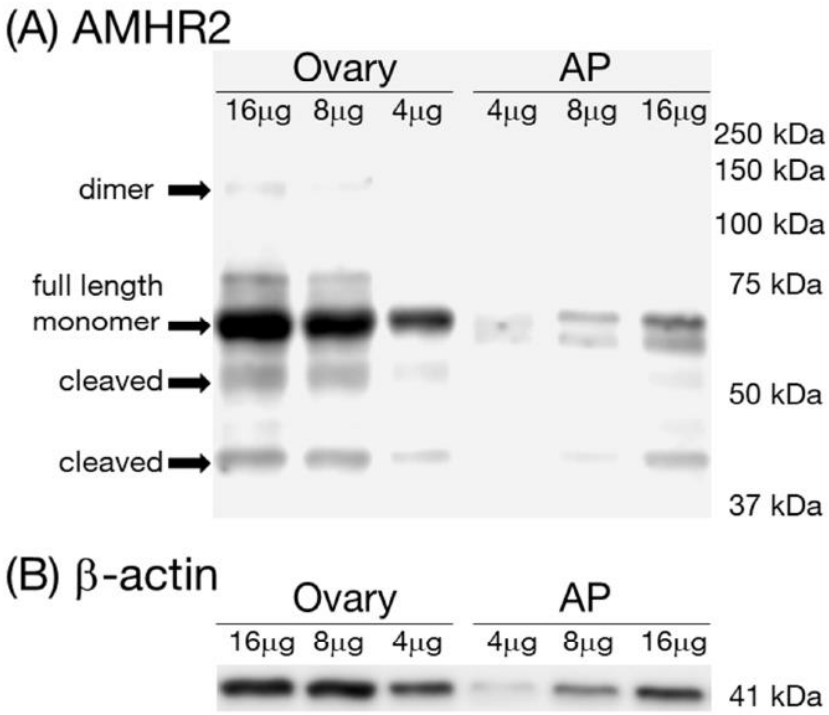

Fig. 2. Results of western blotting using extracts (4, 8, or 16 μg of total protein) from the AP or ovary of post-pubertal heifers and anti-AMHR2 antibody (A) or anti-β–actin antibody (B). We defined bovine AMHR2 bands based on size as dimers, full length monomers, or cleaved monomers, according to previous studies utilizing western blotting for human and mouse AMHR2 (Faure et al. 1996; Hirschhorn et al. 2015).

56x48mm (300 x 300 DPI)

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810

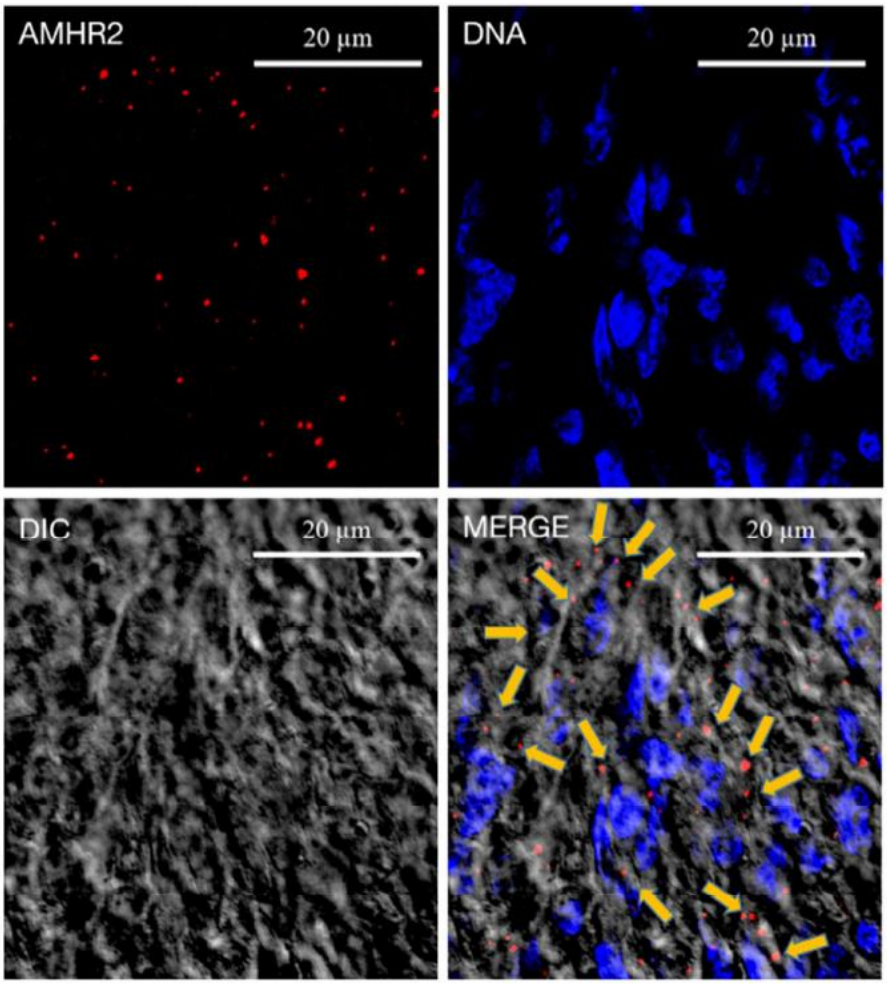

Fig. 3. Fluorescence immunocytochemistry was used to confirm the expression of AMHR2 on the surface of granulosa cells of small (approximately 5 mm) follicles in the ovaries of post-pubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), DNA (dark blue), and differential interference contrast (indicated as DIC). Strong AMHR2 staining appeared to be aggregated (orange arrows), not evenly dispersed. (scale bars = 20 µm)

51x57mm (300 x 300 DPI)

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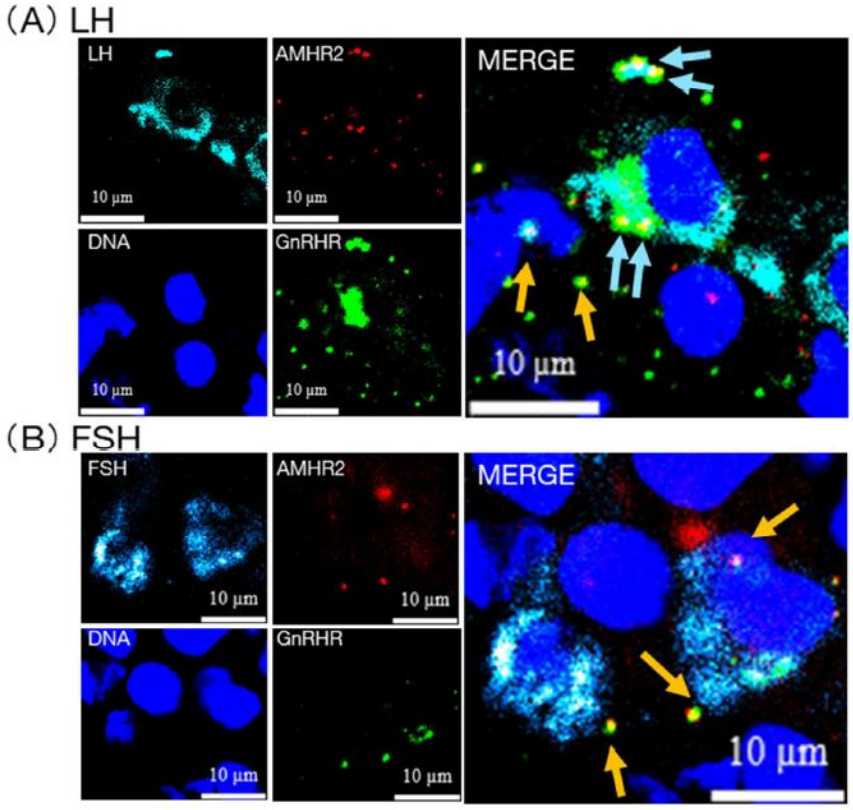


Fig. 4. Triple-fluorescence immunohistochemistry of AP tissue of post-pubertal heifers for AMHR2, gonadotropin-releasing hormone receptor (GnRHR) and either luteinizing hormone (LH) (A) or follicle stimulating hormone (FSH) (B). Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (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.

54x51mm (300 x 300 DPI)

813

814

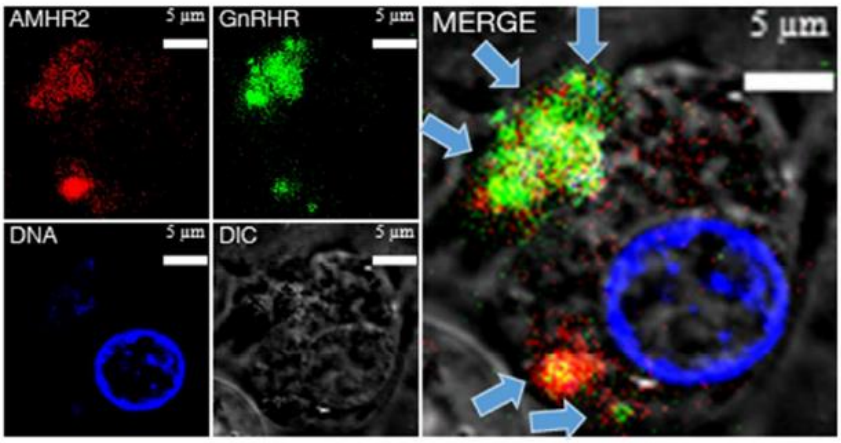
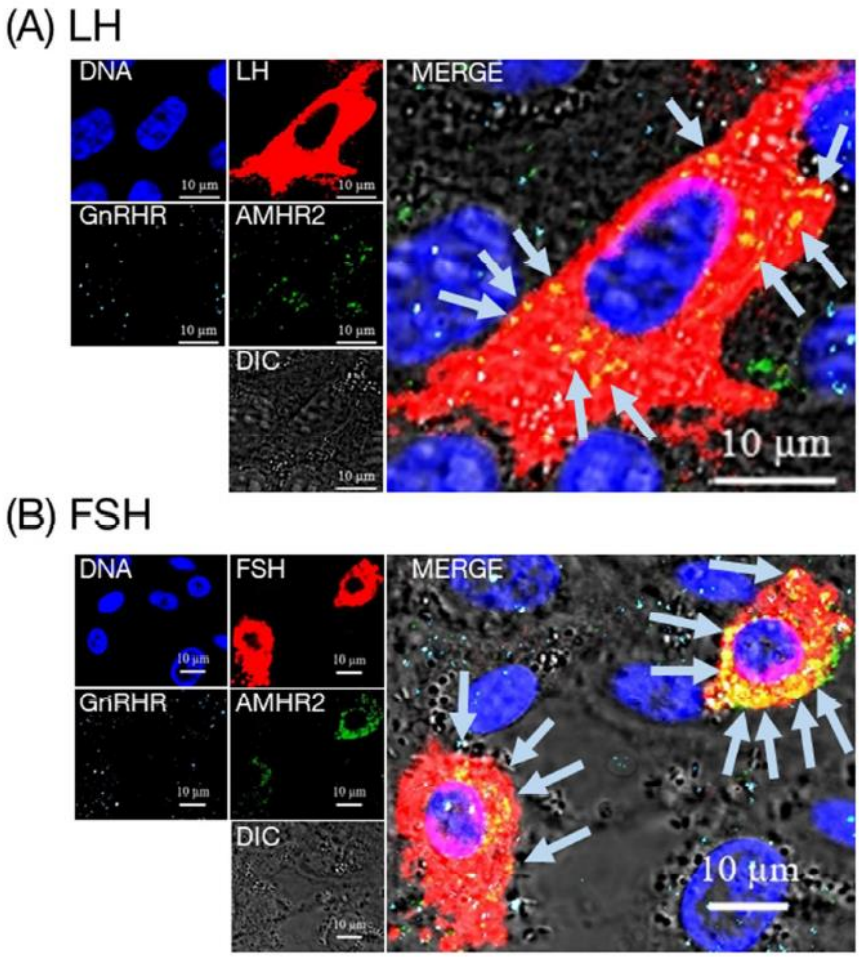


Fig. 5. Fluorescence immunocytochemistry was used to confirm the colocalization (yellow in the merge panel) of AMHR2 and GnRHR on the surface of cultured AP cells (prepared by CellCover method) of post-pubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (green), 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).

38x20mm (300 x 300 DPI)



ig. 6. Triple-fluorescence immunocytochemistry of cultured AP cells (prepared by PFA-Triton method) of post-pubertal heifers for AMHR2, GnRHR and either LH (A) or FSH (B). Images were captured by laser confocal microscopy for AMHR2 (green), GnRHR (light blue) and LH or FSH (red) with counter-staining by DAPI (dark blue). Yellow (shown by arrows) indicates the colocalization of AMHR2 and LH of FSH in LH-positive cells (A) and FSH-positive cells (B). 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. Note that the cells prepared by the PFA-triton method are thinner than those prepared by the CellCover method. Scale bars are 10 µm.

53x59mm (300 x 300 DPI)

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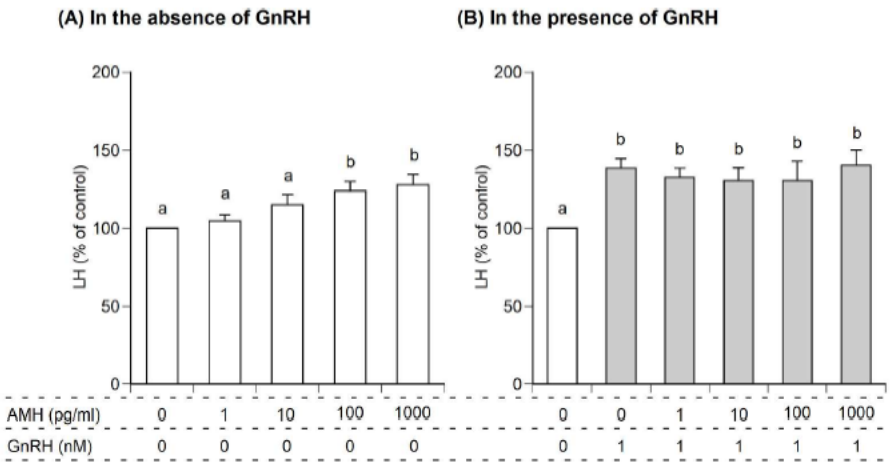
818



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819 226x115mm (300 x 300 DPI)

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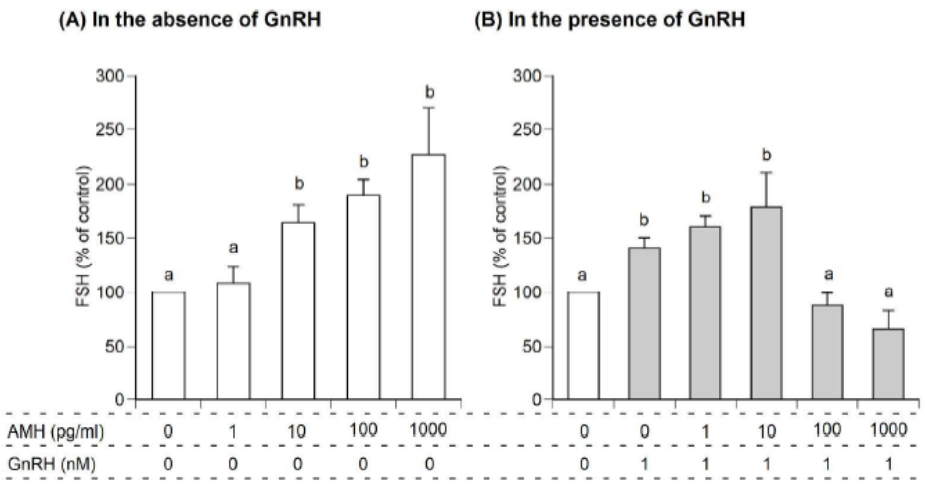

Fig. 8. Comparison of the effects of various concentrations of AMH in media with (A) and without (B) 1 nM GnRH on FSH secretion from cultured AP cells of post-pubertal heifers. The concentrations of FSH in the control cells (cultured in medium alone without AMH and GnRH) were averaged and set at 100%,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

226x115mm (300 x 300 DPI)

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