1 **Anti-Müllerian hormone receptor type 2 is expressed in gonadotrophs of post-**

2 **pubertal heifers to control gonadotropin secretion**

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14 *Running head: AMHR2 controls gonadotropin secretion*

15

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

61   number of high-quality embryos produced by a donor goat or cow (Ireland *et al.* 2008;

62   Monniaux *et al.* 2011). These data suggest the importance of AMH for proper

63   reproductive function in ruminants after puberty.

64   Although the primary role of AMH is at the ovary level in female animals, AMH

65   secreted from preantral and small antral follicles into circulating blood may have roles in

66   other organs. Indeed, the APs of adult rats express mRNA for the main receptor of AMH,

67   AMH receptor type 2 (AMHR2) (Bédécarrats *et al.* 2003). AMH activates LHβ and FSHβ

68   gene expression in LβT2 cells—a murine gonadotroph-derived cell line (Bédécarrats *et*

69   *al.* 2003). Garrel *et al.* (2016) recently reported that AMH stimulates FSH secretion in

70   rats *in vivo*; however, such stimulation is restricted to pre-pubertal female rats. However,

71   there are still no data on the regulatory role of AMH on gonadotropin secretion from

72   gonadotrophs in ruminant species.

73   Gonadotrophs are controlled by GnRH *via* the GnRH receptor (GnRHR) that are

74   present in lipid rafts in the plasma membrane of gonadotrophs (Navratil *et al.* 2009;

75   Wehmeyer *et al.* 2014; Kadokawa *et al.* 2014). The lipid rafts are distinct, relatively

76   insoluble regions that have lower density and are less fluid than surrounding membrane

77   (Simons *et al.* 2000; Head *et al.* 2014), and they facilitate signaling by allowing

78   colocalization of membrane receptors and their downstream signaling components

79   (Simons *et al.* 2000; Head *et al.* 2014). We recently discovered that two orphan receptors,

80   GPR61 and GPR153, are colocalized with GnRHR in gonadotroph plasma membrane

81   lipid rafts (Pandey *et al.* 2017a, 2017b). Therefore, gonadotroph lipid rafts containing

82   GnRHR may contain AMHR2. In the present study, we tested the hypothesis that AMHR2

83   is expressed in the gonadotrophs of post-pubertal heifers to control gonadotropin

84   secretion.

85

86   **Materials and Methods**

87   *AP and ovary sample collection*

88   We obtained AP tissue from post-pubertal (26 months of age) Japanese Black heifers

89   at a local abattoir, using a previously described method (Kadokawa *et al.* 2014). The

90   heifers were in the middle luteal phase, i.e., 8 to 12 days after ovulation, as determined

91   by macroscopic examination of the ovaries and uterus (Miyamoto *et al*. 2000); the AP

92   show the highest LH and GnRHR concentrations in this phase (Nett *et al.* 1987).

93   Granulosa cells in small antral follicles express AMHR2 mRNA (Poole *et al.* 2016).

94   Therefore, we also collected ovary tissue samples from the same heifers to use as positive

95   controls of AMHR2 in western blotting and immunohistochemistry assays.

96   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

114 per AP using ReverTra Ace qPCR RT Master Mix (Toyobo) according to the

115 manufacturer's protocol.

116   In order to determine the expression of AMHR2 mRNA in the AP, PCR was

117 conducted using one of three pairs of primers designed by Primer3 based on reference

118 sequence of bovine AMHR2 [National Center for Biotechnology Information (NCBI)

119 reference sequence of bovine AMHR2 is NM_001205328.1], as one of PCR primers must

120 span exon-exon junction. Table 1 shows the details of the primers, and the expected PCR-

121 product sizes of the AMHR2 were 340 bp, 320 bp, and 277 bp. Using a Veriti 96–Well

122 Thermal Cycler (Thermoscientific), PCR was performed using 20 ng of cDNA and

123 polymerase (Tks Gflex DNA Polymerase, Takara Bio Inc.) under the following

124 thermocycles: 94 °C for 1 min for pre-denaturing followed by 35 cycles of 98°C for 10 s,

125 60°C for 15 s, and 68°C for 30 s. PCR products were separated on 1.5% agarose gel by

126 electrophoresis with a molecular marker [Gene Ladder 100 (0.1-2kbp), Nippon Gene,

127 Tokyo, Japan], stained with fluorescent stain (Gelstar, Lonza, Allendale, NJ), and

128 observed using a charge-coupled device (CCD) imaging system (GelDoc; Bio-Rad,

129 Hercules, CA, US). The PCR products were purified with the NucleoSpin Extract II kit

130 (Takara Bio Inc.) and then sequenced with a sequencer (ABI3130, Thermo Fisher

131 Scientific, Waltham, MA, US) using one of the PCR primers and the Dye Terminator v3.1

132   Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences obtained were used as

133   query terms with which to search the homology sequence in the DDBJ/GenBankTM/EBI

134   Data Bank using the basic nucleotide local alignment search tool (BLAST) optimized for

135   highly similar sequences (available on the NCBI website).

136

137   *Development anti-AMHR2 chicken antibody*

138   We previously determined using the SOSUI v.1.11 algorithm (Hirokawa *et al.* 1998;

139   http://harrier.nagahama-i-bio.ac.jp/sosui/) that bovine AMHR2 protein [543 amino acids;

140   accession number NP_001192257.1 in NCBI reference bovine sequences] contains one

141   hydrophobic transmembrane domains (amino acid 146–168) linked by hydrophilic

142   extracellular and intracellular regions. This structure is the same as the reported structure

143   of mouse AMHR2 (Sakalar *et al.* 2015).

144   Genetyx ver. 11 (Gentyx, Tokyo, Japan) was utilized to predict antigenic determinants

145   based on an algorithm derived by Hopp and Woods (1981). For antibody production, a

146   peptide corresponding to amino acids 31–45 (GVRGSTQNLGKLLDA), an extracellular

147   region that is located near the N terminus of the AMHR2, was used for three reasons.

148   First, this peptide has no homology to the corresponding region of chicken AMHR2

149   (XP_015145444.1). Second, the peptide sequences are in downstream region of the signal

150   peptide of bovine AMHR2 (amino acid 1–17). Third, we could confirm that no other

151   protein encoded in the bovine genome exhibited homology to the peptide sequences of

152   the AMHR2 by comparison with the sequences retrieved from DDBJ/GenBankTM/EBI

153   Data Bank, using the protein BLAST.

154   A commercial service (Scrum Inc., Tokyo, Japan) was utilized for synthesis of antigen

155   peptide (C-GVRGSTQNLGKLLDA), conjugation with keyhole limpet hemocyanin

156   (KLH), immunization, and antibody purification. Briefly, the peptide was synthesized

157   and the purity verified (>99.0%) using high-performance liquid chromatography

158   followed by mass spectrometry. Then, KLH was conjugated to the sulfhydryl group of

159   the cysteine to produce an immunogen that was then emulsified with Complete Freund's

160   adjuvant and injected into chickens five times at 14-day intervals. Blood was collected 7

161   days after the final immunization and the antibody was purified by affinity column

162   chromatography (PD10; GE Healthcare, Amersham, UK) containing an antigen-

163   conjugated gel prepared with the SulfoLink Immobilization Kit (Thermo Scientific).

164

165   *Other antibodies used in this study*

166   We previously developed a guinea pig polyclonal antibody that recognizes the N-

167   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

204   membrane with stripping solution (Nacalai Tesque Inc., Kyoto, Japan), the membrane

205   was used for immunoblotting with the anti-β-actin mouse monoclonal antibody (A2228,

206   1:50,000 dilution; Sigma-Aldrich, St. Louis, MO, USA).

207

208   *Fluorescent immunohistochemistry and confocal microscopic observation*

209   After storage in 4% paraformaldehyde PBS at 4°C for 16 h, the AP (n = 5) or ovary

210   (n = 5) tissue blocks were placed in 30% sucrose PBS until the blocks were infiltrated

211   with sucrose. The methods for immunofluorescence analysis of AP tissue have been

212   described previously (Kadokawa *et al.* 2014). Briefly, we prepared 15-µm sagittal

213   sections and mounted them on slides. The sections were treated with 0.3 % Triton X-100

214   in PBS for 15 min, then, incubated with 0.5 mL of PBS containing 10% normal goat

215   serum (Wako Pure Chemicals, Osaka, Japan) for blocking for 1 h. Incubation with a

216   cocktail of primary antibodies (anti-GnRHR guinea pig antibody, anti-AMHR2 chicken

217   antibody, and either anti-LHβ or anti-FSHβ mouse antibody [all diluted as 1:1,000]) for

218   12 h at 4°C was followed by incubation with a cocktail of fluorochrome-conjugated

219   secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 546 goat anti-

220   mouse IgG, and Alexa Fluor 647 goat anti-guinea pig IgG [all from Thermo Fisher

221   Scientific and diluted as 1 µg/mL]) and 1 µg/mL of 4′, 6′-diamino-2-phenylindole (DAPI;

222 Wako Pure Chemicals) for 2 h at room temperature. Moreover, we prepared 15-µm ovary

223 sections, incubated with anti-AMHR2 chicken antibody (1:1,000), and then incubated

224 with 1 µg/mL Alexa Fluor 488 goat anti-chicken IgG and DAPI to use as positive controls

225 to verify the anti-AMHR2 antibody.

226 The stained sections on slides were observed with a confocal microscope (LSM710;

227 Carl Zeiss, Göttingen, Germany) equipped with diode (405 nm), argon (488 nm), HeNe

228 (533 nm) and HeNe (633 nm) lasers. Images obtained by fluorescence microscopy were

229 scanned with a 40× or 63× oil-immersion objective and recorded by a CCD camera

230 system controlled by ZEN2012 black edition software (Carl Zeiss). GnRHR, AMHR2,

231 and LHβ or FSHβ localization were examined in confocal images of triple-

232 immunolabeled specimens. In the confocal images obtained after immunohistochemistry

233 analysis, the GnRHR is shown in green, AMHR2 is shown in red, and LHβ or FSHβ is

234 shown in light blue. Therefore, the yellow coloration on the surface of light blue-colored

235 cells indicates the colocalization of AMHR2 and GnRHR. The percentage of AMHR2

236 single (red)–labeled light blue-colored cells, or the percentage of double (yellow)-labeled

237 light blue-colored cells, among all of the AMHR2-positive light blue-colored cells (sum

238 of the numbers of red-labeled and yellow-labeled light blue-colored cells), were

239 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 $(2.5 \times 10^5$ cells/mL, total = 0.15 mL per lane) were cultured in the culture medium at

258   37 °C in 5% CO_2 for 82 h, using a microscopy chamber (μ-Slide VI 0.4, Ibidi, Planegg,

259   Germany). We cultured the AP cells for 82 h (3.5 days), as previously described

260   (Hashizume *et al.* 2003; Kadokawa *et al.* 2008; Hashizume *et al.* 2009; Kadokawa *et al.*

261   2014; Nakamura *et al.* 2015). We supplied recombinant human activin A (final

262   concentration, 10 ng/ml; R&D systems, Minneapolis, MN, US) to stimulate FSH

263   synthesis at 24 h prior to fixation. Mature activin A of bovines (NP_776788.1) and ovines

264   (NP_001009458.1) have 100% homology with that of humans (CAA40805.1), and the

265   24 h culture with the same concentration of same recombinant human activin A product

266   stimulates FSH expression in cultured ovine AP cells (Young *et al.* 2008).

267   We fixed and treated the cultured cells using either 4% paraformaldehyde for 3 min

268   followed by 0.1% Triton X-100 treatment for 1 min (PFA-Triton method), or fixation for

269   2 min with CellCover (Anacyte Laboratories UG, Kuhreder, Hamburg), instead of 4%

270   PFA, and no Triton X-100 treatment (CellCover method), as described by Kadokawa *et*

271   *al. (*2014). Briefly, one of the aforementioned methods was used to treat the cells attached

272   to the bottom of the microscopy chamber. For the PFA-Triton method, the fixed cells were

273   incubated with 0.1 mL of the same cocktail of primary antibodies for 2 h at room

274   temperature. Incubation with Triton X-100 allowed both anti-GnRHR and anti-AMHR2

275   antibodies to bind to target proteins in the cytoplasm and at the cell surface. For the

276   CellCover method, the fixed cells were incubated with only guinea pig anti-GnRHR and

277   chicken anti-AMHR2 (both 1:1,000) for 2 h at room temperature. The cells were not

278   treated with Triton X-100, so the antibodies bound only to the extracellular domains of

279   the respective receptors in most cells, although some cytoplasmic labeling occurred in

280   broken cells. For both PFA-Triton and CellCover methods, cells were incubated with

281   fluorochrome-conjugated secondary antibody cocktail and DAPI, and subjected to

282   confocal microscopy to produce fluorescence micrographs and differential interference

283   contrast (DIC) images on a single plane. Signal specificity was confirmed using negative

284   controls in which the primary antiserum was omitted or pre-absorbed with 5 nM antigen

285   peptide, or in which the normal chicken IgG replaced the primary antibody. Eight

286   randomly selected images of cells prepared by CellCover method were analyzed for co-

287   localization utilizing the ZEN 2012 black edition software (Carl Zeiss) to calculate

288   overlap coefficients (Manders *et al.* 1993) for the Alexa Fluor 488 and Alexa Fluor 647

289   fluorophores.

290

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

292   The AP cells derived from 8 heifers were prepared using the protocol described above.

293   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 37ºC 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

330   of detection was 0.40 ng/mL. At 2.04 ng/mL, the intra-assay coefficient of variation was

331   3.6% and inter-assay coefficient of variation was 6.2%. The concentration of FSH was

332   measured in duplicate samples of culture media by double antibody RIA using ^{125}I-

333   labeled bFSH, reference grade bFSH, and anti-oFSH antiserum (AFP5318C, AFP5346D,

334   and AFPC5288113, NIDDK). The limit of detection was 0.20 ng/mL. At 4.00 ng/mL, the

335   intra-assay coefficient of variation was 4.3% and inter-assay coefficient of variation was

336   7.1%.

337

338   *Statistical analysis*

339   The statistical significance of differences in LH or FSH concentration were analyzed

340   by one-factor ANOVA followed by *post-hoc* comparisons using Fisher's protected least

341   significant difference (PLSD) test using StatView version 5.0 for Windows (SAS Institute,

342   Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$. Data are expressed

343   as mean ± standard error of the mean (SEM).

344

345   **Results**

346   *Expression of AMHR2 mRNA in AP of post-pubertal heifers*

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

348   agarose gel after electrophoresis (Fig. 1). Homology searching in the gene databases for

349   the obtained sequence of amplified products using the first, second and third primer pair

350   respectively revealed that the best match alignment was bovine AMHR2

351   (NM_001205328.1), which had a query coverage of 100%, an e-value of 0.0, and a

352   maximum alignment identity of 99%. No other bovine gene was found to have a

353   homology for the obtained sequences of amplified products, leading to the conclusion

354   that the sequences of the amplified products were identical with the sequence of bovine

355   AMHR2.

356

357   *Western blotting for AMHR2*

358   The presence of AMHR2 in the AP and ovarian tissue was analyzed by western

359   blot, using anti-AMHR2 antibody (Fig. 2). The anti-AMHR2 antibody revealed similar

360   bands in the two tissues, with few differences (Fig. 2A). The major difference was that

361   AP tissue showed weaker bands than ovarian tissue did. Nevertheless, β-actin bands

362   showed weaker staining in both tissue types (Fig. 2B). Finally, another difference was

363   that the full-length monomer in the ovary appeared as a single band, whereas in AP cells,

364   it appeared as a doublet (Fig. 2A). No bands were observed in the negative control

365   membranes, where the primary antiserum was pre-absorbed with the antigen peptide.

366

Immunofluorescence analysis of AMHR2 expression in bovine granulosa cells

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

371

Immunofluorescence analysis of AMHR2 expression in bovine AP tissue

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

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

402   AP cells derived from post-pubertal heifers cultured in the absence (A) or presence (B)

403   of GnRH. The effect of different concentrations of AMH was significant (P < 0.05) in the

404   absence of GnRH (Fig. 8A). The wells with 10 pg/ml (P < 0.05), 100 pg/ml (P < 0.05),

405   and 1000 pg/ml (P < 0.05) of AMH, but not 1 pg/ml of AMH, had higher FSH

406   concentrations than those without AMH (8.4 ± 1.2 ng/ml). The effect of different

407   concentrations of AMH was significant (P < 0.05) in the presence of GnRH (Fig. 8B).

408   FSH concentrations in the medium of GnRH wells were higher (P < 0.05) than those in

409   the medium of control wells. There was no effect of 1 pg/ml or 10 pg/ml of AMH on the

410   GnRH-induced FSH secretion. There was a suppressing effect of 100 pg/ml (P < 0.05)

411   and 1000 pg/ml (P < 0.05) of AMH on the GnRH-induced FSH secretion.

412

413   **Discussion**

414   To the best of our knowledge, this study is the first to report that AP cells express

415   AMHR2 in ruminants and that AMH significantly affects LH and FSH secretion from AP

416   cells. Fluorescent immunohistochemistry using the anti-AMHR2 antibody showed the

417   strong signal located on the surface of granulosa cells in small antral follicles, where

418   AMHR2 mRNA is expressed (Poole *et al.* 2016). Therefore, the anti-bovine AMHR2 is

419   the first developed tool that can be used for immunohistochemistry in bovine samples.

420

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,

474   AMH may control also LH secretion, but weakly. Intraperitoneal injection with AMH

475   increases FSH concentration in blood collected 18 h later in rats; however, AMH injection

476   does not significantly increase LH concentration in the same blood samples (Garrel *et al.*

477   2016). Therefore, the effect of AMH on LH secretion *in vivo* may not become significant.

478   It is well known that GPCR proteins can form functionally active homomers and

479   heteromers with different receptors (Ritter and Hall 2009). We obtained the strong

480   positive overlap coefficient between AMHR2 and GnRHR on the cell-surface. This

481   overlap coefficient was greater than that reported between GnRHR and flotillin-1 in

482   cultured LβT2 cells (0.50; Wehmeyer *et al.* 2014) and similar to that we previously found

483   between GnRHR and GPR61 (0.71; Pandey *et al.* 2017a) and GPR153 (0.75; Pandey *et*

484   *al.* 2017b) in bovine gonadotrophs. Heterodimerization among paralogs of GnRHRs of a

485   protochordate results in the modulation of ligand-binding affinity, signal transduction,

486   and internalization (Satake *et al.* 2013). Thus, it is possible that AMHR2 forms a

487   heteromer, affecting ligand-binding affinity, signal transduction, and internalization of

488   GnRHR, and thus the synthesis and secretion of LH and FSH in AP of vertebrates.

489   Furthermore, a recent study (Hossain *et al.* 2016) suggested that GPR61 form heteromers

490   with other GPCRs. Therefore, further studies are required to clarify whether GnRHR form

491   heteromers with GPR61, GPR153, and AMHR2.

492   In this study, we observed multiple, not single, bands of AMHR2 in western

493   blotting, which has been reported previously. For example, Faure *et al.* (1996) reported

494   three bands (82, 73, and 63 kDa) of dimers, full-length monomers, and cleaved monomers.

495   Hirschhorn *et al.* (2015) reported more bands (~58 kDa, ~69 kDa, and ~71 kDa) of dimers,

496   full-length monomers, and cleaved monomers. AMHR2 is present as dimers, full-length

497   monomers, and cleaved monomers in bovine ovaries and APs. Treatment with *N*-

498   glycosidase F shows a further two bands (68 kDa and 61 kDa) by cutting down by

499   approximately 5 and 2 kDa, because AMHR2 is *O*-glycosylated (Faure *et al.* 1996). The

500   full-length monomers in APs appeared as a doublet, whereas those in the ovary appeared

501   as a single band in this study. Therefore, this study suggests that bovine AMHR2 is

502   glycosylated, and the difference in the number of full-length monomers between the AP

503   and ovary might be because of the glycosylation differences.

504   The anti-AMHR2 antibody revealed similar bands in the two tissues in the western

505   blot. However, AP tissue showed weaker bands than ovarian tissue did. Nevertheless, β-

506   actin bands showed weaker staining in both tissue types. This suggests that the AP cell

507   lanes were loaded with a lower amount of proteins than expected. A second difference

508   between AP and ovarian cells was the absence of the dimeric AMHR2 band in AP cells.

509   However, this might be the consequence of the lower protein amount used in the AP cell

510 western blot. In fact, the high molecular weight band was detectable in the ovarian tissue

511 extract only at the highest dose (i.e., 16 µg/lane).

512 　　We found that approximately 20% of AMHR2-positive cells were non-gonadotrophs.

513 At the time of our manuscript preparation, no reports published on AMHR2 in non-

514 gonadotrophs. An AMHR2 polymorphism (482 A>G) was associated with lower

515 prolactin levels in women with polycystic ovary syndrome (Georgopoulos *et al.* 2013).

516 Therefore, lactotrophs may express AMHR2 to play an important role in polycystic ovary

517 syndrome, which is a possibility that bears further consideration in future investigations.

518 　　In conclusion, AMHR2 is expressed in the gonadotrophs of post-pubertal heifers to

519 control gonadotropin secretion.

520

528

Conflicts of Interest

The authors declare no conflicts of interest.

531

References

Bédécarrats, G. Y., O'Neill, F. H., Norwitz, E. R., Kaiser, U. B., and Teixeira, J. (2003). Regulation of gonadotropin gene expression by Mullerian inhibiting substance. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 9348-9353. doi: 10.1073/pnas.1633592100

Belville, C., Van, Vlijmen, H., Ehrenfels, C., Pepinsky, B., Rezaie, A. R., Picard, J. Y., Josso, N., di Clemente, N., and Cate, . RL. (2004). Mutations of the anti-mullerian hormone gene in patients with persistent mullerian duct syndrome: biosynthesis, secretion, and processing of the abnormal proteins and analysis using a three-dimensional model. *Mol. Endocrinol.* **18**, 708-721. doiI: 10.1210/me.2003-0358

Bhide, P., and Homburg, R. (2016). Anti-Müllerian hormone and polycystic ovary syndrome. *Best. Pract. Res. Clin. Obstet. Gynaecol.* **37**, 38-45. doi: 10.1016/j.bpobgyn.2016.03.004

Borromeo, V., Amsterdam, A., Berrini, A., Gaggioli, D., Dantes, A., and Secchi, C. (2004). Characterization of biologically active bovine pituitary FSH purified by

546   immunoaffinity chromatography using a monoclonal antibody. *Gen. Comp.*

547   *Endocrinol.* **139**, 179-189. doi: 10.1016/j.ygcen.2004.09.005

548   Campbell, B.K., Clinton, M., and Webb, R. (2012). The role of anti-Mullerian hormone

549   (AMH) during follicle development in a monovulatory species (sheep).

550   *Endocrinology* **153**, 4533-4543. doi: 10.1210/en.2012-1158.

551   Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R.,

552   Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M.,

553   Visser. J. A., Wallace, W. H., and Anderson, R. A. (2014). The physiology and

554   clinical utility of anti-Mullerian hormone in women. *Hum. Reprod. Update* **20**, 370-

555   385. doi: 10.1093/humupd/dmt062

556   Durlinger, A. L., Gruijters, M. J., Kramer, P., Karels, B., Kumar, T. R., Matzuk, M. M.,

557   Rose, U. M., de Jong, F. H., Uilenbroek, J.T., Grootegoed, J.A., and Themmen, A.

558   P. (2001). Anti-Mullerian hormone attenuates the effects of FSH on follicle

559   development in the mouse ovary. *Endocrinology* **142**, 4891-4899. doi:

560   10.1210/endo.142.11.8486

561   El-Sheikh Ali, H., Kitahara, G., Nibe, K., Yamaguchi, R., Horii, Y., Zaabel, S., and Osawa,

562   T. (2013). Plasma anti-Müllerian hormone as a biomarker for bovine granulosa-

563   theca cell tumors: Comparison with immunoreactive inhibin and ovarian steroid

564 concentrations. *Theriogenology* **80**, 940-949. doi:

565 10.1016/j.theriogenology.2013.07.022

566 Faure, E., Gouédard, L., Imbeaud, S., Cate, R., Picard, J. Y., Josso, N., and di Clemente,

567 N. (1996). Mutant isoforms of the anti-Müllerian hormone type II receptor are not

568 expressed at the cell membrane. *J. Biol. Chem.* **271**, 30571-30575. doi:

569 10.1074/jbc.271.48.30571

570 Garrel, G., Racine, C., L'Hôte, D., Denoyelle, C., Guigon, C. J., di Clemente, N., and

571 Cohen-Tannoudji, J. (2016). Anti-Müllerian hormone: a new actor of sexual

572 dimorphism in pituitary gonadotrope activity before puberty. *Sci. Rep.* **6**, 23790. doi:

573 10.1038/srep23790

574 Georgopoulos, N. A., Karagiannidou, E., Koika, V., Roupas, N. D., Armeni, A., Marioli,

575 D., Papadakis, E., Welt, C. K., and Panidis, D. (2013). Increased frequency of the

576 anti-mullerian-inhibiting hormone receptor 2 (AMHR2) 482 A>G polymorphism in

577 women with polycystic ovary syndrome: relationship to luteinizing hormone levels.

578 *J. Clin. Endocrinol. Metab.* **98**, E1866-E1870. doi: 10.1210/jc.2013-2458

579 Hashizume, T., Horiuchi, M., Tate, N., Nonaka, S., Kojima, M., Hosoda, H., and

580 Kangawa, K. (2003). Effects of ghrelin on growth hormone secretion from cultured

581 adenohypophysial cells in cattle. *Endocr. J.* **50**, 289-295. doi:

582　　　　10.1507/endocrj.50.289

583　　Hashizume, T., Onodera, Y., Shida, R., Isobe, E., Suzuki, S., Sawai, K., Kasuya, E., and

584　　　　Nagy, G. M. (2009). Characteristics of prolactin-releasing response to salsolinol

585　　　　(SAL) and thyrotropin-releasing hormone (TRH) in ruminants. *Domest. Anim.*

586　　　　*Endocrinol.* **36**, 99-104. doi: 10.1016/j.domaniend.2008.11.001

587　　Head, B. P., Patel, H. H., and Insel, P. A. (2014). Interaction of membrane/lipid rafts with

588　　　　the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators

589　　　　of cytoskeletal arrangement and cell signaling. *Biochim. Biophys. Acta.* **1838**, 532-

590　　　　545. doi: 10.1016/j.bbamem.2013.07.018

591　　Hernandez-Medrano, J. H., Campbell, B. K., and Webb, R. (2012). Nutritional influences

592　　　　on folliculogenesis. *Reprod. Dom. Anim. Suppl.* **4**, 274-282. doi: 10.1111/j.1439-

593　　　　0531.2012.02086

594　　Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and

595　　　　secondary structure prediction system for membrane proteins. *Bioinformatics* **14**,

596　　　　378-379. doi: 10.1093/bioinformatics/14.4.378

597　　Hirschhorn, T., di Clemente, N., Amsalem, A. R., Pepinsky, R. B., Picard, J. Y.,

598　　　　Smorodinsky, N. I., Cate, R. L., and Ehrlich, M. (2015). Constitutive negative

599　　　　regulation in the processing of the anti-Müllerian hormone receptor II. *J. Cell Sci.*

600 **128**, 1352-1364. doi: 10.1242/jcs.160143

601 Hopp, T. P., and Woods, K. R. (1981). Prediction of protein antigenic determinants from

602 amino acid sequences. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3824-3828. doi:

603 10.1073/pnas.78.6.3824

604 Hossain, M. S., Mineno, K., Katafuchi, T. (2016). Neuronal orphan G-protein coupled

605 receptor proteins mediate plasmalogens-induced activation of ERK and Akt

606 signaling. *PLoS One* **11**, e0150846. doi: 10.1371/journal.pone.0150846

607 Iqbal, J., Latchoumanin, O., Sari, I. P., Lang, R. J., Coleman, H. A., Parkington, H. C.,

608 and Clarke, I. J. (2009). Estradiol-17beta inhibits gonadotropin-releasing hormone-

609 induced Ca2+ in gonadotropes to regulate negative feedback on luteinizing hormone

610 release. *Endocrinology* **150**, 4213-4220. doi: 10.1210/en.2009-0092

611 Ilha, G. F., Rovani, M. T., Gasperin, B.G., Ferreira, R., de Macedo, M. P., Neto, O. A.,

612 Duggavathi, R., Bordignon, V., and Goncalves, P. B. (2016). Regulation of Anti-

613 Mullerian Hormone and Its Receptor Expression around Follicle Deviation in Cattle.

614 *Reprod. Domest. Anim.* **51**,188-194. doi: 10.1111/rda.12662

615 Ireland, J. L., Scheetz, D., Jimenez-Krassel, F., Themmen, A. P., Ward, F., Lonergan, P.,

616 Smith, G. W., Perez, G. I., Evans, A. C., and Ireland, J. J. (2008). Antral follicle

617 count reliably predicts number of morphologically healthy oocytes and follicles in

618   ovaries of young adult cattle. *Biol. Reprod.* **79**, 1219-1225. doi:

619   10.1095/biolreprod.108.071670

620   Kadokawa, H., Suzuki, S., and Hashizume, T. (2008). Kisspeptin-10 stimulates the

621   secretion of growth hormone and prolactin directly from cultured bovine anterior

622   pituitary cells. *Anim. Reprod. Sci.* **105**, 404-408. doi:

623   10.1016/j.anireprosci.2007.11.005

624   Kadokawa, H., Pandey, K., Nahar, A., Nakamura, U., and Rudolf, F. O. (2014).

625   Gonadotropin-releasing hormone (GnRH) receptors of cattle aggregate on the

626   surface of gonadotrophs and are increased by elevated GnRH concentrations. *Anim.*

627   *Reprod. Sci.* **150**, 84-95. doi: 10.1016/j.anireprosci.2014.09.008

628   Koizumi, M., Nahar, A., Yamabe, R., and Kadokawa, H. (2016). Positive correlations of

629   age and parity with plasma concentration of macrophage migration inhibitory factor

630   in Japanese black cows. *J. Reprod. Dev.* **62**, 257-263. doi: 10.1262/jrd.2015-144

631   Koizumi, M., and Kadokawa, H. (2017). Positive correlations of age and parity with

632   plasma anti-Müllerian hormone concentrations in Japanese Black cows. *J. Reprod.*

633   *Dev.* **63**, 205-209. doi: 10.1262/jrd.2016-088

634   Kushnir, V. A., Seifer, D. B., Barad, D. H., Sen, A., and Gleicher, N. (2017). Potential

635   therapeutic applications of human anti-Müllerian hormone (AMH) analogues in

636 reproductive medicine. *J. Assist. Reprod. Genet.* **34**, 1105-1113. doi:

637   10.1007/s10815-017-0977-4

638 Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993). Measurement of co-localization

639   of objects in dual-colour confocal images. *J. Microscopy* **169**, 375-382. doi:

640   10.1111/j.1365-2818.1993.tb03313.x

641 Martin, T. L., Fogwell, R. L., and Ireland, J. J. (1991). Concentrations of inhibins and

642   steroids in follicular fluid during development of dominant follicules in heifers. *Biol.*

643   *Reprod.* **44**, 693-700. doi: 10.1095/biolreprod44.4.693

644 Matteri, R. L., Roser, J. F., Baldwin, D. M., Lipovetsky, V., and Papkoff, H. (1987).

645   Characterization of a monoclonal antibody which detects luteinizing hormone from

646   diverse mammalian species. *Domest. Anim. Endocrinol.* **4**, 157-165. doi:

647   10.1016/0739-7240(87)90011-7

648 Miyamoto, Y., Skarzynski, D.J., Okuda, K. (2000). Is tumor necrosis factor alpha a trigger

649   for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in

650   cattle? *Biol. Reprod.* **62**, 1109-1115. doi: 10.1095/biolreprod62.5.1109

651 Monniaux, D., Baril, G., Laine, A. L., Jarrier, P., Poulin, N., Cognié, J., and Fabre, S.

652   (2011). Anti-Mullerian hormone as a predictive endocrine marker for embryo

653   production in the goat. *Reproduction* **142**, 845-854. doi: 10.1530/REP-11-0211

654 Nakamura, U., Rudolf, F.O., Pandey, K., and Kadokawa, H. (2015). The non-steroidal

655 mycoestrogen zeranol suppresses luteinizing hormone secretion from the anterior

656 pituitary of cattle via the estradiol receptor GPR30 in a rapid, non-genomic manner.

657 *Anim. Reprod. Sci.* 1**56**, 118-127. doi: 10.1016/j.anireprosci.2015.03.009

658 Navratil, A. M., Song, H., Hernandez, J. B., Cherrington, B. D., Santos, S. J., Low, J. M.,

659 Do, M. H., and Lawson, M. A. (2009). Insulin augments gonadotropin-releasing

660 hormone induction of translation in LbetaT2 cells. *Mol. Cell. Endocrinol.* **311**, 47-

661 54. doi: 10.1016/j.mce.2009.07.014

662 Nett, T.M., Cermak, D., Braden, T., Manns, J., and Niswender, G. (1987). Pituitary

663 receptors for GnRH and estradiol, and pituitary content of gonadotropins in beef

664 cows. I. Changes during the estrous cycle. *Domest. Anim. Endocrinol.* **4**, 123-132.

665 doi: 10.1016/0739-7240(87)90006-3

666 Nussey, S. S., and Whitehead, S. A. (2001). The pituitary gland. In 'Endocrinology: an

667 integrated approach.' (Eds. S. S. Nussey and S. A. Whitehead) pp. 283-334. (BIOS

668 scientific publishers, Oxford, UK.)

669 Pals, K., Roudbaraki, M., and Denef, C., (2008). Growth hormone-releasing hormone

670 and glucocorticoids determine the balance between luteinising hormone (LH) beta-

671 and LH beta/follicle-stimulating hormone beta-positive gonadotrophs and

672   somatotrophs in the 14-day-old rat pituitary tissue in aggregate cell culture. *J.*

673   *Neuroendocrinol.* **20**, 535-548. doi: 10.1111/j.1365-2826.2008.01698.x

674 Pandey, K., Nahar, A., and Kadokawa, H. (2016). Method for isolating pure bovine

675   gonadotrophs from anterior pituitary using magnetic nanoparticles and anti-

676   gonadotropin-releasing hormone receptor antibody. *J. Vet. Med. Sci.* **78**, 1699-1702.

677   doi: 10.1292/jvms.16-0157

678 Pandey, K., Kereilwe, O., Borromeo, V., and Kadokawa, H. (2017a). Heifers express G-

679   protein coupled receptor 61 in anterior pituitary gonadotrophs in stage-dependent

680   manner. *Anim. Reprod. Sci.* **181**, 93-102. doi: 10.1016/j.anireprosci.2017.03.020

681 Pandey, K., Kereilwe, O., and Kadokawa, H. (2017b). Heifers express G-protein coupled

682   receptor 153 in anterior pituitary gonadotrophs in stage-dependent manner. *Anim.*

683   *Sci. J.* (in press)

684 Pfeiffer, K. E., Jury, L. J., and Larson, J. E. (2014). Determination of anti-Müllerian

685   hormone at estrus during a synchronized and a natural bovine estrous cycle. *Domest.*

686   *Anim. Endocrinol.* **46**, 58-64. doi: 10.1016/j.domaniend.2013.05.004

687 Poole, D. H., Ocón-Grove, O. M., and Johnson, A. L. (2016). Anti-Müllerian hormone

688   (AMH) receptor type II expression and AMH activity in bovine granulosa cells.

689   *Theriogenology* **86**, 1353-1360. doi: 10.1016/j.theriogenology.2016.04.078

690   Ribeiro, E. S., Bisinotto, R. S., Lima, F. S., Greco, L. F., Morrison, A., Kumar, A.,

691   Thatcher, W. W., and Santos, J. E. (2014). Plasma anti-Müllerian hormone in adult

692   dairy cows and associations with fertility. *J. Dairy Sci.* **97**, 6888-6900. doi:

693   10.3168/jds.2014-7908

694   Rico, C., Médigue, C., Fabre, S., Jarrier, P., Bontoux, M., Clément, F., and Monniaux, D.

695   (2011). Regulation of anti-Müllerian hormone production in the cow: a multiscale

696   study at endocrine, ovarian, follicular, and granulosa cell levels. *Biol. Reprod.* **84**,

697   560-571. doi: 10.1095/biolreprod.110.088187

698   Ritter, S. L., and Hall, R. A. (2009). Fine-tuning of GPCR activity by receptor-interacting

699   proteins. *Nat. Rev. Mol. Cell. Biol.* **10**, 819–830. doi: 10.1038/nrm2803

700   Rocha, R. M., Lima, L. F., Carvalho, A. A., Chaves R. N., Bernuci, M. P., Rosa-e-Silva,

701   A. C., Rodrigues, A. P., Campello, C. C., and Figueiredo, J. R. (2016).

702   Immunolocalization of the Anti-Müllerian Hormone (AMH) in Caprine Follicles

703   and the Effects of AMH on In Vitro Culture of Caprine Pre-antral Follicles Enclosed

704   in Ovarian Tissue. *Reprod. Domest. Anim.* **51**, 212-219. doi: 10.1111/rda.12668

705   Sakalar, C., Mazumder, S., Johnson, J. M., Altuntas, C. Z., Jaini, R., Aguilar, R., Naga

706   Prasad, S. V., Connolly, D. C., and Tuohy, V. K. (2015). Regulation of Murine

707   Ovarian Epithelial Carcinoma by Vaccination against the Cytoplasmic Domain of

708   Anti-Müllerian Hormone Receptor II. *J. Immunol. Res.* **2015**, 630287. doi:

709   10.1155/2015/630287

710   Satake, H., Matsubara, S., Aoyama, M., Kawada, T., and Sakai, T. (2013). GPCR

711   heterodimerization in the reproductive system: functional regulation and

712   implication for biodiversity. *Front. Endocrinol. (Lausanne)* **4**, 1-8. doi:

713   10.3389/fendo.2013.00100. eCollection 2013

714   Seifer, D. B., and Merhi, Z. (2014). Is AMH a regulator of follicular atresia? *Assist.*

715   *Reprod. Genet.* **31**, 1403-1407. doi: 10.1007/s10815-014-0328-7

716   Simons, K., and Tooter, D., (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol.*

717   *Cell Biol.* **1**, 31-39. doi: 10.1038/35036052

718   Suzuki, S., Kadokawa, H., and Hashizume, T. (2008). Direct kisspeptin-10 stimulation

719   on luteinizing hormone secretion from bovine and porcine anterior pituitary cells.

720   *Anim. Reprod. Sci.* **103**, 360-365. doi: 10.1016/j.anireprosci.2007.05.016

721   Townsend, J., Sneddon, C. L., and Tortonese, D. J. (2004). Gonadotroph heterogeneity,

722   density and distribution, and gonadotroph-lactotroph associations in the pars distalis

723   of the male equine pituitary gland. *J. Neuroendocrinol.* **16**, 432-440. doi:

724   10.1111/j.1365-2826.2004.01174.x

725   Visser, J. A., and Themmen, A. P. (2014). Role of anti-Mullerian hormone and bone

726   morphogenetic proteins in the regulation of FSH sensitivity. *Mol. Cell. Endocrinol.*

727   **382**, 460-465. doi: 10.1016/j.mce.2013.08.012

728   Wehmeyer, L., Du Toit, A., Lang, D. M., and Hapgood, J. P. (2014). Lipid raft- and

729   protein kinase C-mediated synergism between glucocorticoid- and gonadotropin-

730   releasing hormone signaling results in decreased cell proliferation. *J. Biol. Chem.*

731   **289**, 10235-10251. doi: 10.1074/jbc.M113.544742

732   Young, J. M., Juengel, J. L., Dodds, K. G., Laird, M., Dearden, P. K., McNeilly, A. S.,

733   McNatty, K. P., and Wilson, T. (2008). The activin receptor-like kinase 6 Booroola

734   mutation enhances suppressive effects of bone morphogenetic protein 2 (BMP2),

735   BMP4, BMP6 and growth and differentiation factor-9 on FSH release from ovine

736   primary pituitary cell cultures. *J. Endocrinol.* **196**, 251-261. doi: 10.1677/JOE-07-

737   0148.

738

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	

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744 **Figure Legends**
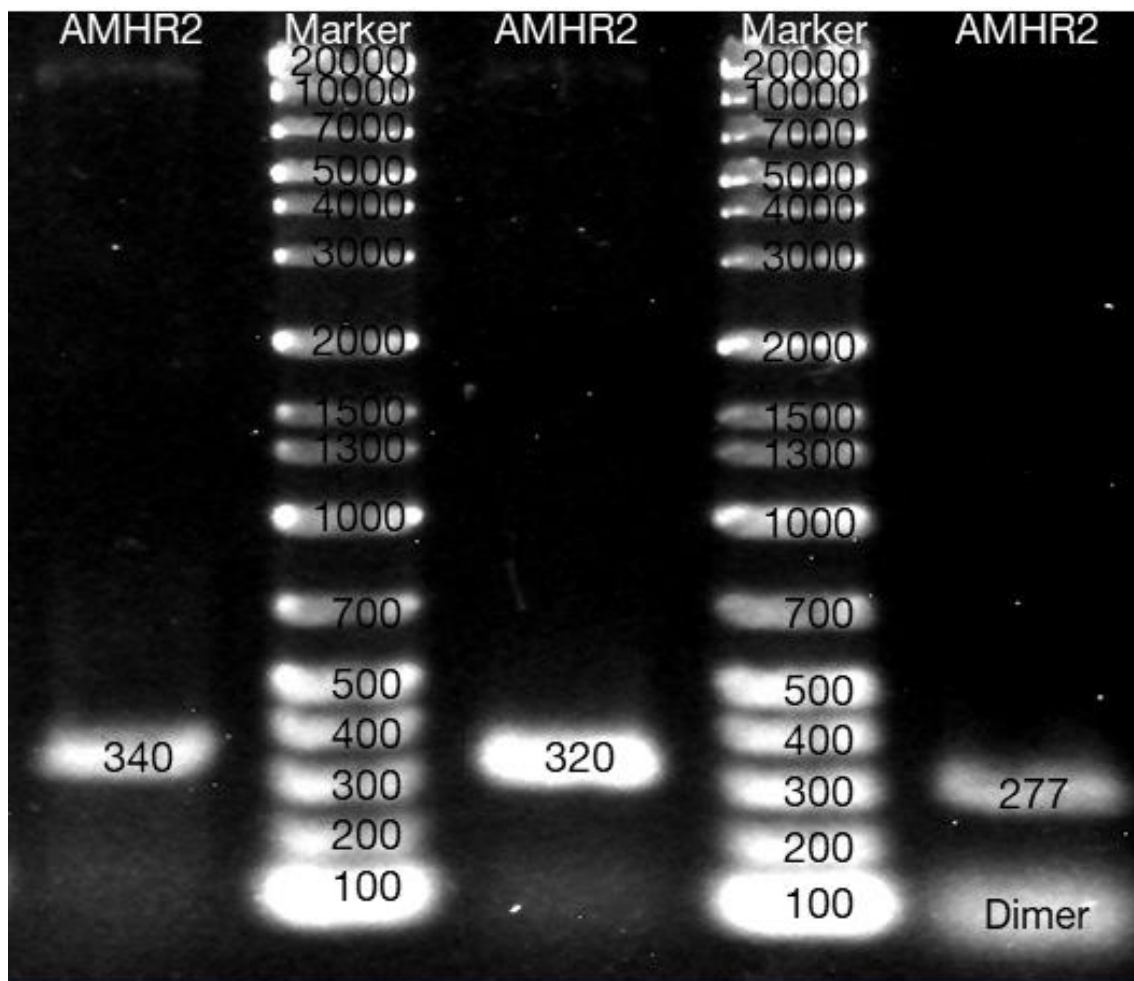
745

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.

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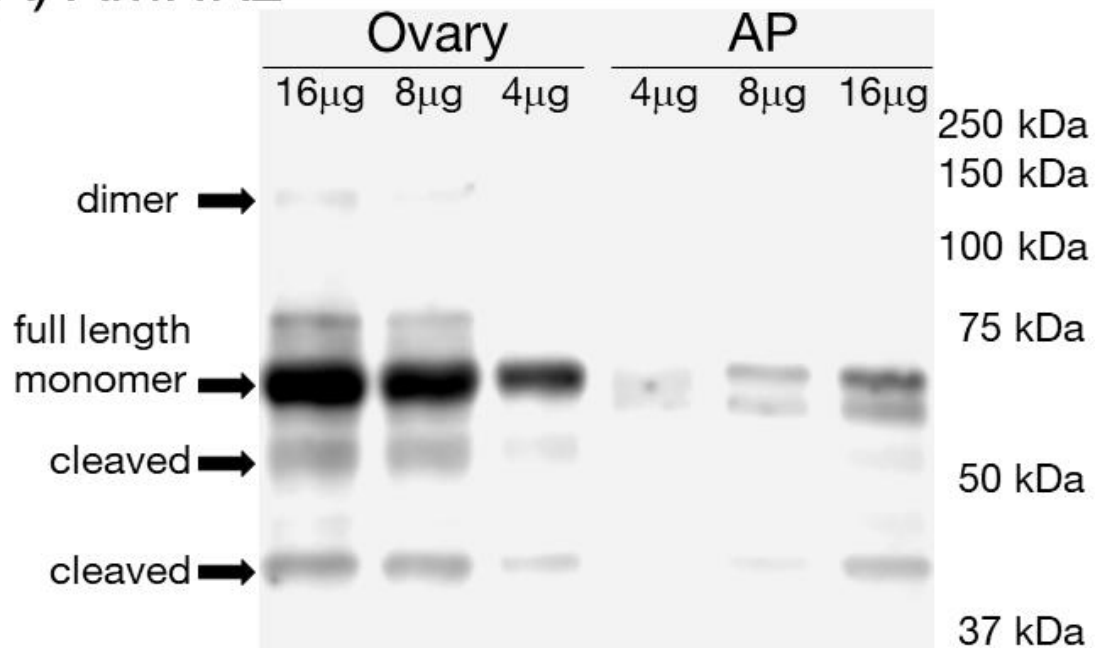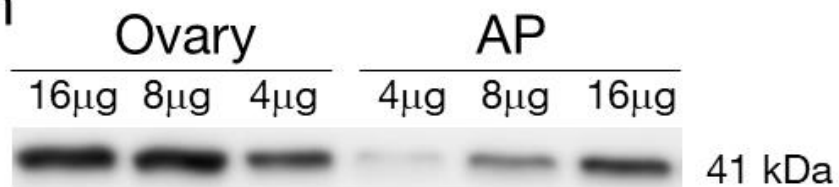
Fig. 2. Results of western blotting using extracts (4, 8, or 16 μg of total protein) from the AP or ovary of post-pubertal heifers and anti-AMHR2 antibody (A) or anti-β−actin antibody (B). We defined bovine AMHR2 bands based on size as dimers, full length monomers, or cleaved monomers, according to previous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

762
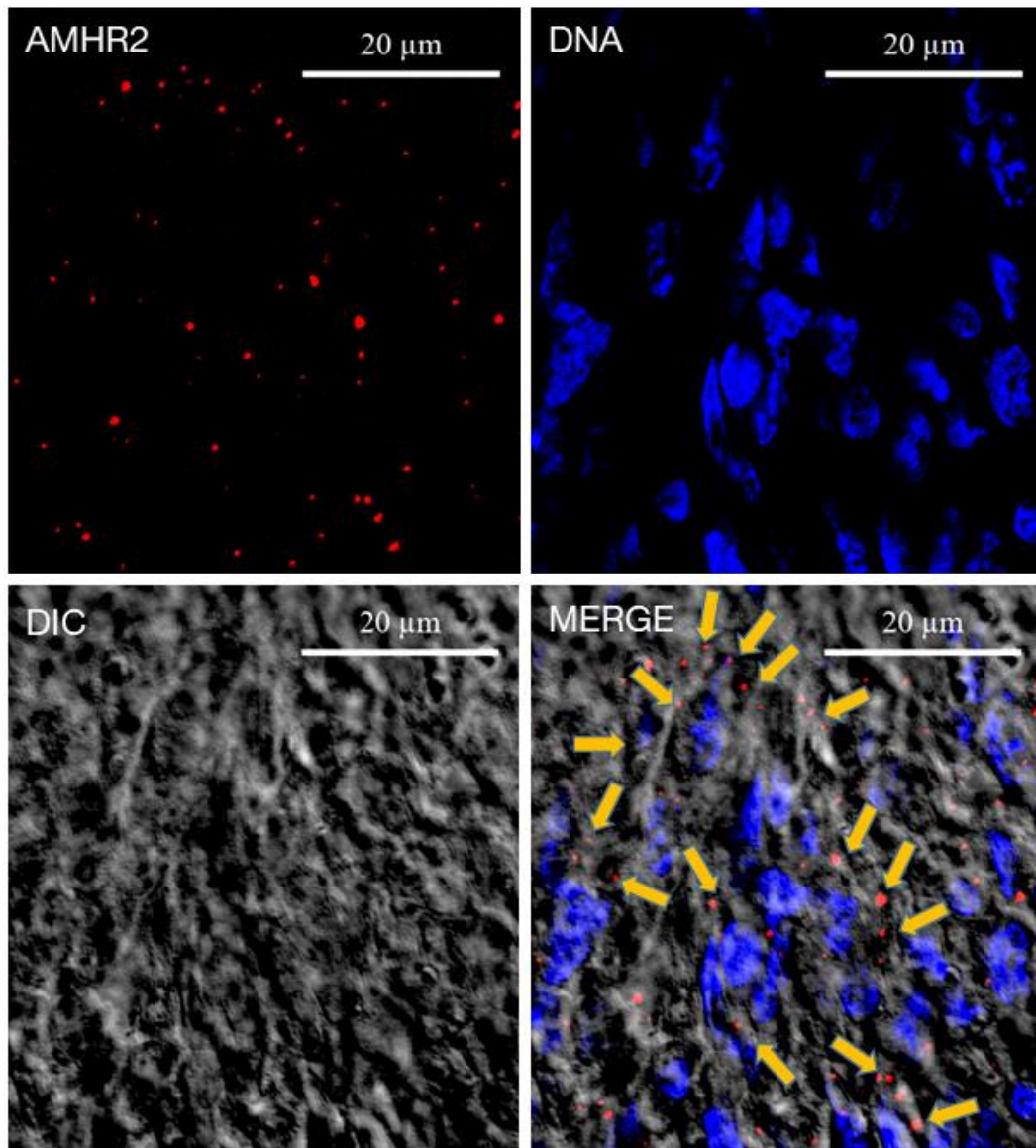
Fig. 3. Fluorescence immunocytochemistry was used to confirm the expression of AMHR2 on the surface of granulosa cells of small (approximately 5 mm) follicles in the ovaries of post-pubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), DNA (dark blue), and differential interference contrast (indicated as DIC). Strong AMHR2 staining appeared to be aggregated (orange arrows), not evenly dispersed. (scale bars = 20 μm)

769
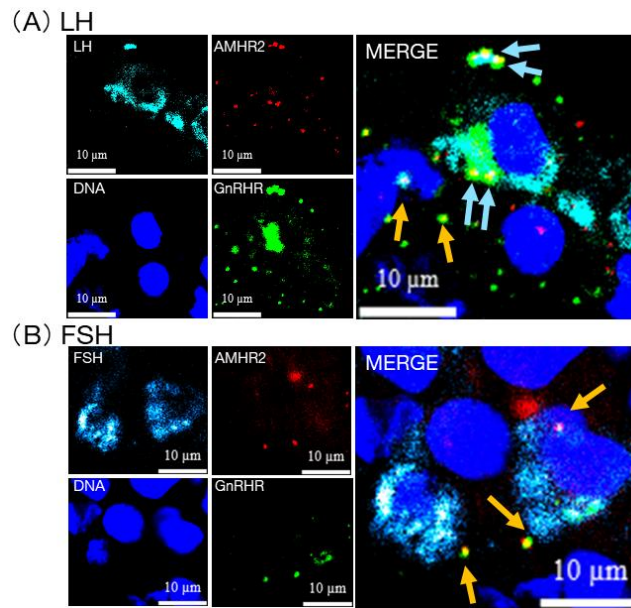
Fig. 4. Triple-fluorescence immunohistochemistry of AP tissue of post-pubertal heifers

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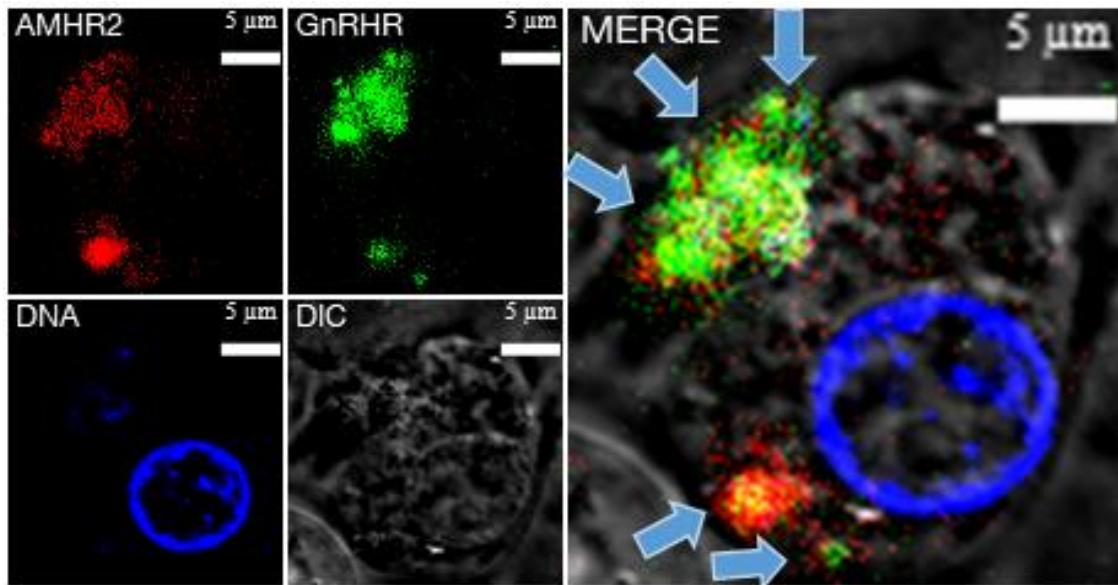
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784   **Fig. 5.** Fluorescence immunocytochemistry was used to confirm the colocalization

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

786   (prepared by CellCover method) of post-pubertal heifers. Images were captured by laser

787   confocal microscopy for AMHR2 (red), GnRHR (green), DNA (dark blue), and DIC on

788   cultured AP cells which did not receive Triton X-100 treatment for antibody penetration.

789   Thus, antibody could only bind AMHR2 and GnRHR on the surface of gonadotrophs.

790   The blue arrows indicate the colocalization of aggregated GnRHR and aggregated

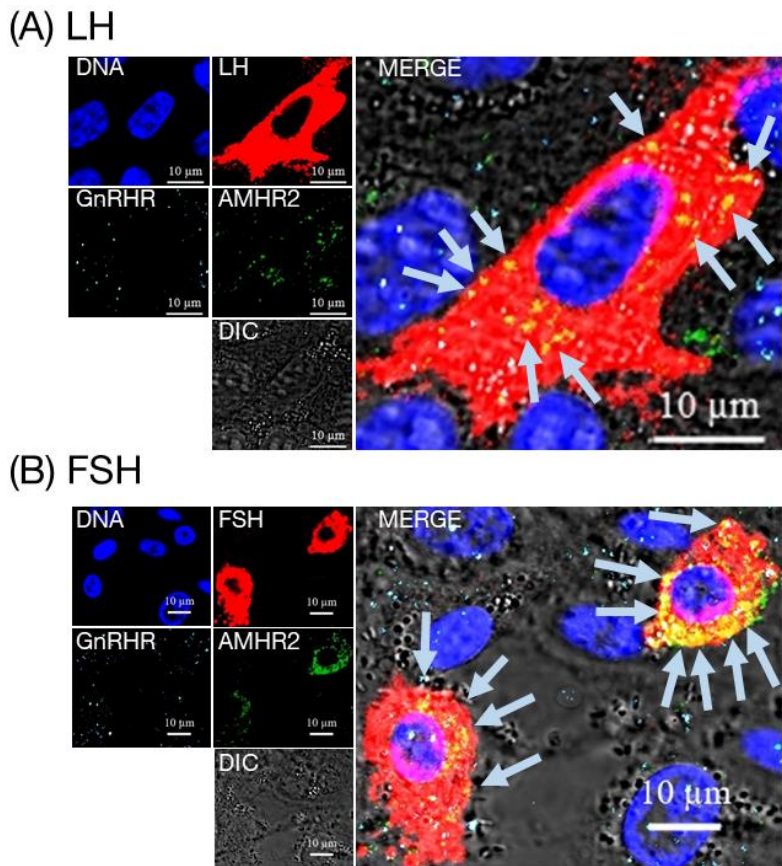791   AMHR2. (scale bars = 5 μm).

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Fig. 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.

(A) In the absence of GnRH   **(B) In the presence of GnRH**
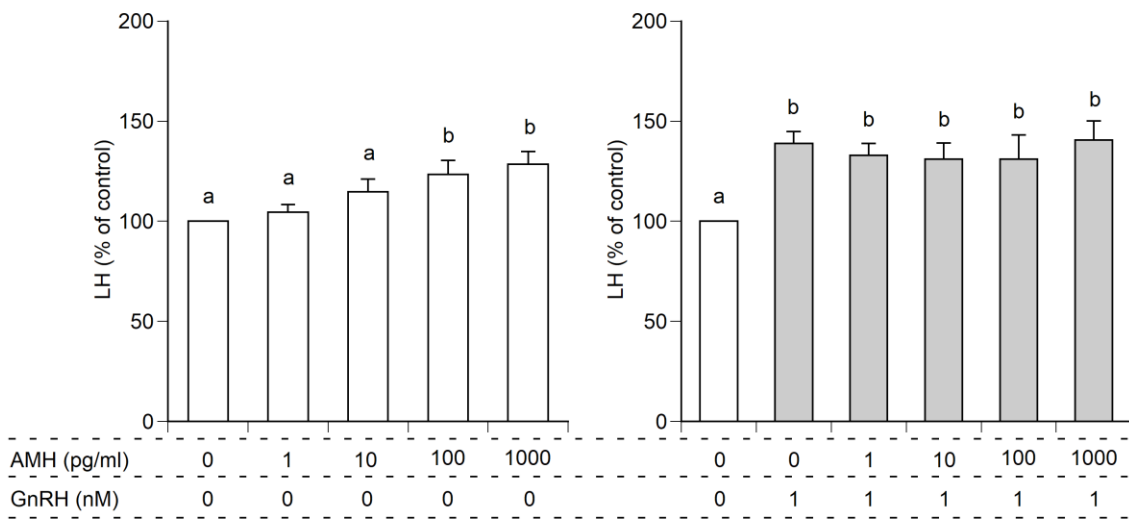
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810 **Fig. 7.** Comparison of the effects of various concentrations of AMH in media with (A)

811 and without (B) 1 nM GnRH on LH secretion from cultured AP cells of post-pubertal

812 heifers. The concentrations of LH in the control cells (cultured in medium alone without

813 AMH and GnRH) were averaged and set at 100%, and the mean LH concentration for

814 each treatment group is expressed as a percentage of the control value. Different letters
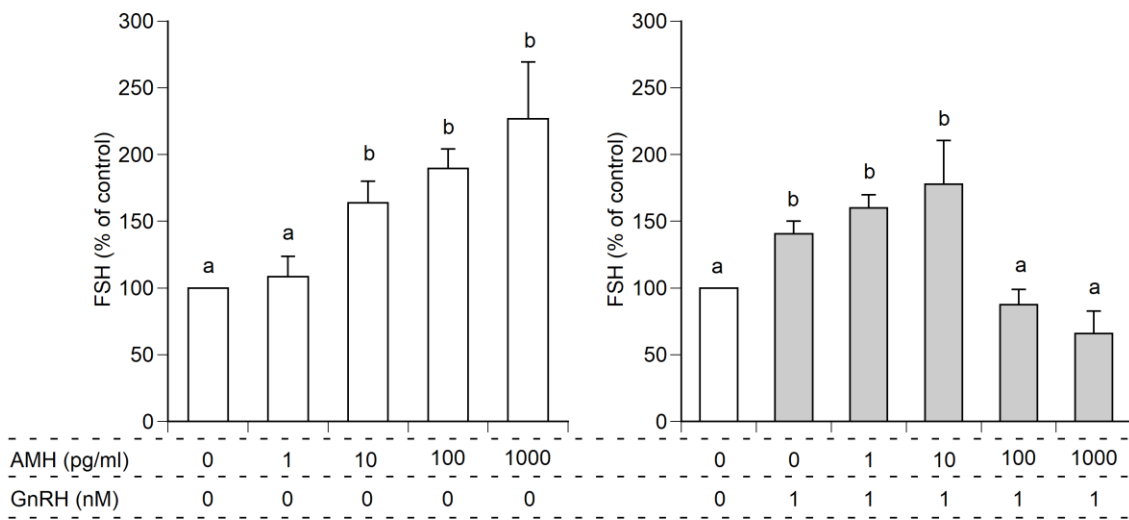
815 indicate statistical differences ($P < 0.05$).

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(A) In the absence of GnRH

(B) In the presence of GnRH

AMH (pg/ml)	0	1	10	100	1000		0	0	1	10	100	1000
GnRH (nM)	0	0	0	0	0		0	1	1	1	1	1

820

821 **Fig. 8.** Comparison of the effects of various concentrations of AMH in media with (A)

822 and without (B) 1 nM GnRH on FSH secretion from cultured AP cells of post-pubertal

823 heifers. The concentrations of FSH in the control cells (cultured in medium alone without

824 AMH and GnRH) were averaged and set at 100%, and the mean FSH concentration for

825 each treatment group is expressed as a percentage of the control value. Different letters

826 indicate statistical differences (P < 0.05).