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Anti-Müllerian hormone receptor type 2 is expressed in gonadotrophs of postpubertal heifers to control gonadotrophin secretion

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Abstract. Preantral and small antral follicles may secret anti-Müllerian hormone (AMH) to control gonadotrophin secretion from ruminant gonadotrophs. The present study investigated whether the main receptor for AMH, AMH receptor type 2 (AMHR2), is expressed in gonadotrophs of postpubertal heifers to control gonadotrophin secretion. Expression of *AMHR2* mRNA was detected in anterior pituitaries (APs) of postpubertal heifers using reverse transcription–polymerase chain reaction. An anti-AMHR2 chicken antibody was developed against the extracellular region near the N-terminus of bovine AMHR2. Western blotting using this antibody detected the expression of AMHR2 protein in APs. Immunofluorescence microscopy using the same antibody visualised colocalisation of AMHR2 with gonadotrophin-releasing hormone (GnRH) receptor on the plasma membrane of gonadotrophs. AP cells were cultured for 3.5 days and then treated with increasing concentrations (0, 1, 10, 100, or 1000 pg mL^{-1}) of AMH. AMH (10–1000 pg mL^{-1}) stimulated ($P < 0.05$) basal FSH secretion. In addition, AMH (100–1000 pg mL^{-1}) weakly stimulated ($P < 0.05$) basal LH secretion. AMH (100–1000 pg mL^{-1}) inhibited GnRH-induced FSH secretion, but not GnRH-induced LH secretion, in AP cells. In conclusion, AMHR2 is expressed in gonadotrophs of postpubertal heifers to control gonadotrophin secretion.

Additional keywords: AMHR2, gonadotrophin-releasing hormone (GnRH) receptor, Müllerian-inhibiting substance, ruminant.

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Introduction

Gonadotrophs in the anterior pituitary (AP) secrete gonadotrophins, LH and FSH to regulate follicle growth, ovulation and corpus luteum (CL) formation in ovaries of vertebrates. Acting as a feedback mechanism, antral follicles and CL secrete steroids and inhibin to control gonadotrophin 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 gonadotrophin secretion from the AP. We question whether preantral and small antral follicles are a silent majority in ovaries.

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein in the transforming growth factor TGF β (TGFβ) family and, in humans and animals, AMH is primarily produced by granulosa cells of preantral and small antral follicles (Bhide and Homburg 2016). AMH regulates follicular development during the gonadotrophin-responsive phase (Hernandez-Medrano *et al.* 2012) and inhibits follicular atresia (Seifer and Merhi 2014). Blood AMH concentrations are indicative of ovarian aging in women (Dewailly *et al.* 2014; Bhide and Homburg 2016). Plasma AMH concentrations are positively correlated 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 level of the ovary in female animals, AMH secreted from preantral and small antral follicles into circulating blood may have actions 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 prepubertal female rats. There are still no data on the regulatory role of AMH

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Table 1. Details of the three primers used for polymerase chain reaction to detect anti-Müllerian hormone receptor type 2 (*AMHR2*) mRNA in bovine anterior pituitaries

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

on gonadotrophin secretion from gonadotrophs in ruminant species.

Gonadotrophs are controlled by gonadotrophin-releasing hormone (GnRH) via the GnRH receptor (GnRHR), present in lipid rafts in the plasma membrane of gonadotrophs (Navratil *et al.* 2009; Kadokawa *et al.* 2014; Wehmeyer *et al.* 2014). The lipid rafts are distinct, relatively insoluble regions that have lower density and are less fluid than the surrounding membrane (Simons and Tooter 2000; Head *et al.* 2014), and they facilitate signalling by allowing colocalisation of membrane receptors and their downstream signalling components (Simons and Tooter 2000; Head *et al.* 2014). We recently discovered that two orphan receptors, namely GPR61 and GPR153, are colocalised with GnRHR in gonadotroph plasma membrane lipid rafts (Pandey *et al.* 2017, 2018). 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 postpubertal heifers to control gonadotrophin secretion.

Materials and methods

AP and ovary sample collection

AP tissue was obtained from postpubertal (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. 8–12 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 for AMHR2 in western blotting and immunohistochemical 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 ($n = 5$) were fixed in 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.

Reverse transcription–polymerase chain reaction, 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) according to the manufacturer's instructions. The extracted RNA samples were treated with ribonuclease-free deoxyribonuclease (Toyobo) to eliminate possible genomic DNA contamination. Using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), the concentration and purity of each RNA sample were evaluated to ensure that the ratio of absorbance at 260/280 nm (A_{260}/A_{280}) was in the acceptable range of 1.8–2.1. The mRNA quality of all samples was verified by electrophoresis of total RNA followed by staining with ethidium bromide, and the *28S* : *18S* ratios were 2 : 1. The cDNA was synthesised from 0.5 μg total RNA per AP using ReverTra Ace qPCR RT Master Mix (Toyobo) according to the manufacturer's instructions.

In order to determine the expression of *AMHR2* mRNA in the AP, polymerase chain reaction (PCR) was conducted using one of three pairs of primers designed by Primer3 based on the reference sequence of bovine *AMHR2* (National Center for Biotechnology Information (NCBI) reference NM_001205328.1), as one of PCR primers must span an exon–exon junction. The primer pairs are listed in Table 1; the expected sizes of the PCR products of *AMHR2* were 340, 320 and 277 bp. Using a Veriti 96-Well Thermal Cycler (Thermo Scientific), PCRs were performed using 20 ng cDNA and polymerase (Tks Gflex DNA Polymerase; Takara Bio) under the following thermocycles: 94°C for 1 min for predenaturing, 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 gels by electrophoresis with a molecular marker (Gene Ladder 100 (0.1–2 kbp); Nippon Gene), stained with fluorescent stain (Gelstar; Lonza) and observed using a charge-coupled device (CCD) imaging system (GelDoc; Bio-Rad). The PCR products were purified using a NucleoSpin Extract II kit (Takara Bio) and then sequenced with a sequencer (ABI3130; Thermo Fisher Scientific) using one of the PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences obtained were used as query terms with which to search for homology sequences in the DDBJ/GenBank/EBI data bank using the basic nucleotide local alignment search tool (BLAST) optimised for highly similar sequences (available on the NCBI website).

Development anti-AMHR2 chicken antibody

Previously, using the SOSUI v.1.11 algorithm (Hirokawa *et al.* 1998; http://harrier.nagahama-i-bio.ac.jp/sosui/, accessed 10 March 2016 , we determined that bovine AMHR2 protein (543 amino acids; Accession no. NP_001192257.1 in NCBI reference bovine sequences) contains one hydrophobic transmembrane domains (amino acids 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) was used 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 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 a region downstream of the signal peptide of bovine AMHR2 (amino acids 1–17). Third, we confirmed that no other protein encoded in the bovine genome exhibited homology with the peptide sequences of the AMHR2 by comparison with sequences retrieved from the DDBJ/GenBankTM/EBI data bank using BLAST.

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

Other antibodies used in the present study

We previously developed a guinea pig polyclonal antibody that recognises the N-terminal extracellular domain (corresponding to amino acids 1–29; MANSDSPEQNENHCSAINSSIPLTPGSLP) of GnRHR (anti-GnRHR). The specificity of the anti-GnRHR antibody was verified by western blotting, and pretreatment with anti-GnRHR antibody inhibited GnRH-induced LH secretion from cultured bovine gonadotrophs (Kadokawa *et al.* 2014). In addition, we previously used the anti-GnRHR antibody for immunofluorescence detection of GnRHR in the plasma membrane of bovine gonadotrophs (Kadokawa *et al.* 2014; Pandey *et al.* 2016). We observed a strong and localised GnRHR-positive staining signal as an aggregation on the plasma membrane of gonadotrophs (Kadokawa *et al.* 2014). In the present study, we used the anti-GnRHR antibody and a mouse monoclonal anti-LHβ antibody (Clone 518-B7; Matteri *et al.* 1987) for immunohistochemical analysis of AP tissue and cultured AP cells. This anti-LH-β antibody does not cross-react with other pituitary hormones (Iqbal *et al.* 2009). We also used a mouse monoclonal anti-FSHβ subunit antibody (Clone A3C12) that does not cross-react with other pituitary hormones (Borromeo *et al.* 2004) for immunohistochemical analysis of AP tissue.

Western blotting for AMHR2

Briefly, protein was extracted from AP ($n = 3$) or ovary ($n = 3$; positive control) samples and western blotting was performed as previously described (Kadokawa *et al.* 2014). The extracted protein (33.4 μg total protein in 37.5 μL) was mixed in 12.5 μL of 4× Laemmli sample buffer (Bio-Rad) containing 10% (v/v) β-mercaptoethanol, then boiled for 3 min at 100°C. Boiled protein samples were quickly cooled in ice, then 4, 8 or 16 μg total protein was loaded onto sodium dodecyl sulfate–polyacrylamide gels, along with a molecular weight marker (Precision Plus Protein All Blue Standards; Bio-Rad) for resolution by electrophoresis at 100 V for 90 min. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting with the anti-AMHR2 chicken antibody (1 : 25 000 dilution) after blocking with 0.1% Tween 20 and 5% non-fat dry milk for 1 h at 25°C. Membranes were incubated overnight at 4°C with the primary antibody, washed with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20 and then incubated with horseradish peroxidase (HRP)-conjugated anti-chicken IgG goat antibody (Bethyl Laboratories; 1 : 50 000 dilution) at 25°C for 1 h. Protein bands were visualised using an ECL-Prime chemiluminescence kit (GE Healthcare) and CCD imaging system (Fujifilm). Previous studies using western blotting for AMHR2 reported that human and mouse AMHR2 are present as dimers, full-length monomers or cleaved monomers (Faure *et al.* 1996; Hirschhorn *et al.* 2015). Thus, we defined bovine AMHR2 bands based on mobility as one of these structure types. After antibodies had been removed from the PVDF membrane with stripping solution (Nacalai Tesque), the membrane was used for immunoblotting with the anti-β-actin mouse monoclonal antibody (A2228; 1 : 50 000 dilution; Sigma-Aldrich).

Fluorescent immunohistochemistry and confocal microscopic observation

After storage in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) at 4°C for 16 h, the AP ($n = 5$) or ovary ($n = 5$) tissue blocks were placed in 30% sucrose–PBS until the blocks were infiltrated with sucrose. The methods for immunofluorescence analysis of AP tissue have been described previously (Kadokawa *et al.* 2014). Briefly, we prepared 15-μm sagittal sections and mounted them on slides. The sections were treated with 0.3% Triton X-100 in PBS for 15 min, then incubated with 0.5 mL PBS containing 10% normal goat serum (Wako Pure Chemicals) for blocking for 1 h. Incubation with a cocktail of primary antibodies (anti-GnRHR guinea pig antibody, anti-AMHR2 chicken antibody and either anti-LHβ or anti-FSHβ mouse antibody; all diluted 1 : 1000) for 12 h at 4°C was followed by incubation with a cocktail of fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 647 goat anti-guinea pig IgG; all from Thermo Fisher Scientific; all diluted 1 μg mL^{-1}) and 1 μg mL^{-1} 4′,6′-diamino-2-phenylindole (DAPI; Wako Pure Chemicals) for 2 h at room

temperature. In addition, 15-μm ovary sections were prepared, incubated with anti-AMHR2 chicken antibody (1 : 1000 dilution) and then incubated with 1 μg mL^{-1} Alexa Fluor 488 goat anti-chicken IgG and DAPI as positive controls to verify the anti-AMHR2 antibody.

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

AP cells from five heifers were dispersed enzymatically using the method of Suzuki *et al.* (2008), and cell viability was confirmed to be >90% by Trypan blue exclusion. Total cell yield was $19.8 \pm 0.8 \times 10^6$ (mean ± s.e.m.) cells per pituitary gland. The dispersed cells were then suspended in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) containing 1× non-essential amino acids (Thermo Fisher Scientific), 100 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 10% horse serum (Thermo Fisher Scientific) and 2.5% fetal bovine serum (Thermo Fisher Scientific). The cells (2.5×10^5 cells mL^{-1}; total = 0.15 mL per lane) were cultured in the culture medium at 37°C in 5% CO_2 for 82 h in a microscopy chamber (μ-Slide VI 0.4; Ibidi). AP cells were cultured for 82 h (3.5 days), as described previously (Hashizume *et al.* 2003, 2009; Kadokawa *et al.* 2008, 2014; Nakamura *et al.* 2015). Recombinant human activin A (final concentration 10 ng mL^{-1}; R&D Systems) was supplied to stimulate FSH synthesis 24 h before fixation. Mature activin A of bovine (NP_776788.1) and ovine (NP_001009458.1) has 100% homology with that of humans activin A (CAA40805.1) and 24-h culture with the same concentration of the same recombinant human activin A product stimulated FSH expression in cultured ovine AP cells (Young *et al.* 2008).

Cultured cells were either fixed using 4% paraformaldehyde for 3 min followed by 0.1% Triton X-100 treatment for 1 min (PFA-Triton method) or fixed for 2 min with CellCover (Anacyte Laboratories), instead of 4% PFA, without subsequent Triton X-100 treatment (CellCover method), as described by Kadokawa *et al.* (2014). Briefly, one of these two methods was used to treat the cells attached to the bottom of the microscopy chamber. For the PFA-Triton method, the fixed cells were incubated with 0.1 mL of the same cocktail of primary antibodies for 2 h at room temperature. Incubation with Triton X-100 allowed both anti-GnRHR and anti-AMHR2 antibodies to bind to target proteins in the cytoplasm and at the cell surface. In the case of the CellCover method, the fixed cells were incubated for 2 h at room temperature with guinea pig anti-GnRHR and chicken anti-AMHR2 only (both 1 : 1000 dilution). The cells were not treated with Triton X-100, so the antibodies bound only to the extracellular domains of the respective receptors in most cells, although some cytoplasmic labelling occurred in broken cells. For both the PFA-Triton and CellCover methods, cells were incubated with fluorochrome-conjugated secondary antibody cocktail and DAPI, and subjected to confocal microscopy to produce fluorescence micrographs and differential interference contrast (DIC) images on a single plane. Signal specificity was confirmed using negative controls in which the primary antiserum was omitted or preabsorbed with 5 nM antigen peptide, or in which the normal chicken IgG replaced the primary antibody. Eight randomly selected images of cells prepared by the CellCover method were analysed for colocalisation using ZEN 2012 black edition software (Carl Zeiss) to calculate overlap coefficients (Manders *et al.* 1993) for the Alexa Fluor 488 and Alexa Fluor 647 fluorophores.

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

The AP cells derived from eight heifers were prepared using the protocol described above. After the cells (2.5×10^5 cells mL^{-1}; total 0.3 mL) had been plated in 48-well culture plates (Sumitomo Bakelite), they were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 82 h. Recombinant human activin A (final concentration 10 ng mL^{-1}) was supplied to stimulate FSH synthesis 24 h before the AMH test.

In the test to evaluate the effects of AMH in the absence of GnRH, the old medium was replaced by 295 μL DMEM containing 0.1% bovine serum albumin (BSA) and 10 ng mL^{-1} activin A and cells were incubated for 2 h. Cells were treated with the addition of either 5 μL DMEM alone or 5 μL DMEM containing different concentrations of human recombinant AMH (R&D Systems; final concentration 0, 1, 10, 100 or 1000 pg mL^{-1} AMH).

The bioactive region in the C-terminal region of mature AMH (Belville *et al.* 2004) of bovines (NP_776315.1) and goat (XP_017906255.1) has 96% homology with that of human mature AMH (NP_000470.2), and the same recombinant human AMH product has a biological effect on goat follicles (Rocha *et al.* 2016).

After incubation for a further 2 h, the medium from each well was collected for radioimmunoassay (RIA) analysis of LH and FSH levels. In a previous study, we found that the physiological

concentration of AMH in blood in Japanese Black cows ranged between 5 and 300 pg mL^{-1} (Koizumi and Kadokawa 2017). The concentrations of AMH used in the present study were based on these findings.

In experiments evaluating the effects of AMH in the presence of GnRH, the old medium was replaced with 290 μL DMEM containing 0.1% BSA and 10 ng mL^{-1} activin A and cells were incubated at 37°C for 2 h. Cells were pretreated by the addition of 5 μL DMEM alone or 5 μL DMEM containing different concentrations (0, 60, 600, 6000 and 60 000 pg mL^{-1}) of human recombinant AMH. Cells were incubated with gentle shaking for 5 min and then treated with 5 μL of 60 nM GnRH (Peptide Institute) dissolved in DMEM for 2 h in order to stimulate LH and FSH secretion. The pretreatment plus the GnRH treatment yielded a final concentration of 0, 1, 10, 100, or 1000 pg mL^{-1} AMH. The final concentration of GnRH was 1 nM in all treatments (Kadokawa *et al.* 2014), except in the control. Control wells were treated with 5 μL DMEM, but were not incubated with GnRH. 'GnRH' wells were pretreated with 5 μL DMEM for 5 min and were then incubated with GnRH for 2 h. After incubation for 2 h, the medium from each well was collected for LH and FSH RIA.

RIA to measure gonadotrophin concentrations in culture media

The concentration of LH was measured in duplicate samples of culture media by double-antibody RIA using ^{125}I-labelled bovine (b) LH and anti-ovine (o) LH-antiserum (AFP11743B and AFP192279 respectively; National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)). The limit of detection was 0.40 ng mL^{-1}. At 2.04 ng mL^{-1}, the intra- and interassay CVs were 3.6% and 6.2% respectively. The concentration of FSH was measured in duplicate samples of culture media by double-antibody RIA using ^{125}I-labelled bFSH, reference-grade bFSH and anti-oFSH antiserum (AFP5318C, AFP5346D and AFPC5288113 respectively; NIDDK). The limit of detection was 0.20 ng mL^{-1}. At 4.00 ng mL^{-1}, the intra- and interassay CVs were 4.3% and 7.1% respectively.

Statistical analysis

The significance of differences in LH or FSH concentrations was analysed by one-factor analysis of variance (ANOVA) followed by post hoc comparisons using Fisher's protected least significant difference (PLSD) test using StatView version 5.0 for Windows (SAS Institute). The level of significance was set at $P < 0.05$. Data are expressed as the mean ± s.e.m.

Results

Expression of AMHR2 *mRNA in the AP of postpubertal heifers*

The expected PCR products (sizes 340, 320 and 277 bp) were observed in the agarose gel after electrophoresis (Fig. 1). Homology searching in the gene databases for the obtained sequences of amplified products using the first, second and third primer pairs 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*.

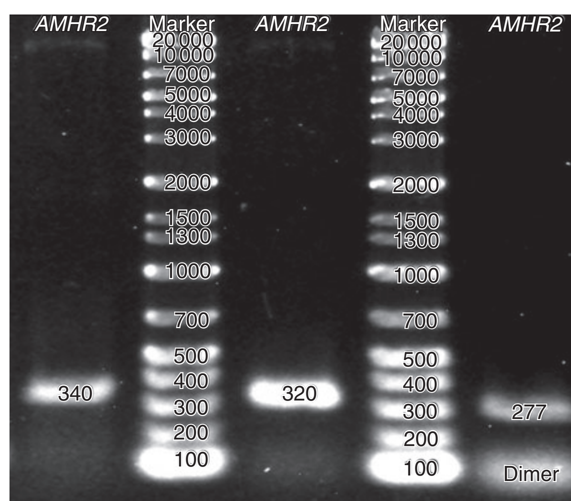

Fig. 1. Expression of anti-Müllerian hormone (AMH) receptor type 2 (*AMHR2*) mRNA, as detected by reverse transcription–polymerase chain reaction. Electrophoresis of polymerase chain reaction (PCR)-amplified DNA products using one of three pairs of primers for bovine *AMHR2* and cDNA derived from anterior pituitary (AP) of postpubertal heifers. The lanes labelled *AMHR2* demonstrate that the DNA products obtained were of the size that had been expected, namely 340, 320 and 277 bp. The other two lanes labelled 'Marker' are the DNA markers.

Western blotting for AMHR2

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

Immunofluorescence analysis of AMHR2 expression in bovine granulosa cells

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

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).

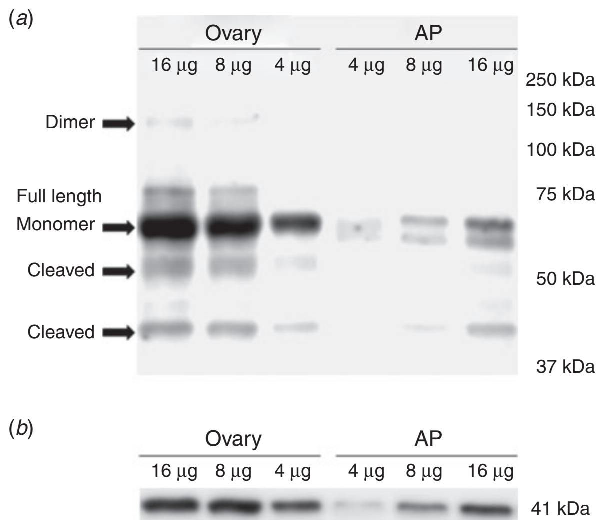

Fig. 2. Results of western blotting using extracts 4, 8 or 16 µg total protein from the anterior pituitary (AP) or ovary of postpubertal heifers and (*a*) anti-Müllerian hormone receptor type 2 (AMHR2) antibody or (*b*) anti-β-actin antibody. Bovine AMHR2 bands were defined as dimers, full-length monomers or cleaved monomers based on size, according to previous studies using western blotting for human and mouse AMHR2 (Faure *et al.* 1996; Hirschhorn *et al.* 2015).

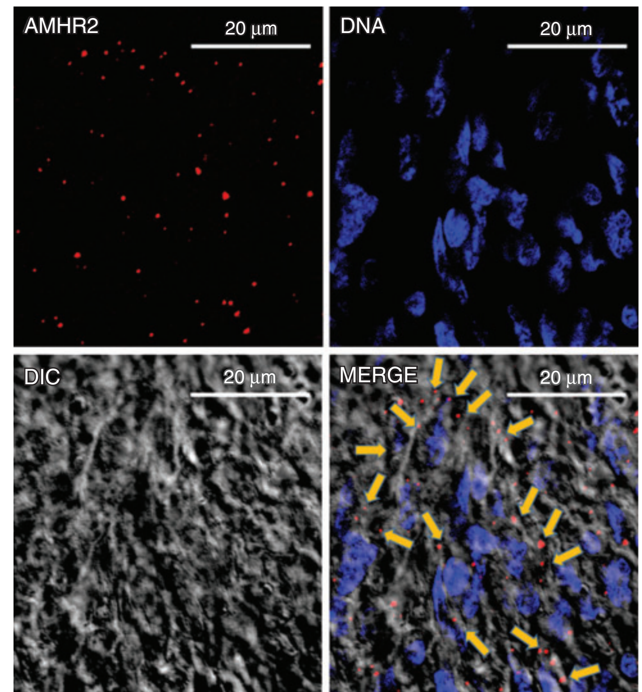

Fig. 3. Fluorescence immunocytochemistry was used to confirm the expression of anti-Müllerian hormone receptor type 2 (AMHR2) on the surface of granulosa cells of small (~5 mm) follicles in the ovaries of postpubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), DNA (dark blue) and differential interference contrast (DIC). Strong AMHR2 staining appeared to be aggregated (arrows), not evenly dispersed.

AMHR2 and GnRHR colocalised in the majority of both LHβ-positive (Fig. 4*a*) and FSHβ-positive (Fig. 4*b*) cells. The focus depth of the high magnification lens used in the present study is thin, thus the best focus for GnRHR and AMHR2 on plasma membrane was quite different from the best focus for both the nucleus and cytoplasmic LHβ or FSHβ. Thus, we know that both membrane receptors are on the cell surface. The percentage of single- and double-labelled AMHR2- and GnRHR-positive cells was 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 ± 3.2% of GnRHR-positive cells were AMHR2 positive, whereas 78.4 ± 1.8% of AMHR2-positive cells were GnRHR positive.

AMHR2 and GnRHR aggregate on the surface of cultured AP cells

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

AMHR2 expression in cultured gonadotrophs

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

Effects of AMH on gonadotrophin secretion from cultured AP cells

Fig. 7 shows the effects of various concentrations of AMH on LH secretion from AP cells derived from postpubertal heifers cultured in the absence (Fig. 7*a*) or presence (Fig. 7*b*) of GnRH. In the absence of GnRH (Fig. 7*a*), 100 and 1000 pg mL^{-1} AMH increased ($P < 0.05$) LH secretion compared with control (medium LH concentration of control group was 17.6 ± 2.4 ng mL^{-1}). Conversely, AMH had no effect on GnRH-induced LH secretion (Fig. 7*b*).

Fig. 8 shows the effects of different concentrations of AMH on FSH secretion from AP cells derived from postpubertal heifers cultured in the absence (Fig. 8*a*) or presence (Fig. 8*b*) of GnRH. The effect of different concentrations of AMH was significant ($P < 0.05$) in the absence of GnRH (Fig. 8*a*). FSH concentrations were significantly ($P < 0.05$) higher in wells with 10, 100 and 1000 pg mL^{-1} AMH, but not 1 pg mL^{-1} AMH, than those without AMH (8.4 ± 1.2 ng mL^{-1}). The effects of different concentrations of AMH were significant ($P < 0.05$) in the presence of GnRH (Fig. 8*b*). FSH concentrations in the medium of GnRH-containing wells were higher ($P < 0.05$) than those in the medium of control wells. AMH at 1 or 10 pg mL^{-1} had no effect on GnRH-induced FSH secretion. However, 100 and 1000 pg mL^{-1} AMH suppressed GnRH-induced FSH secretion ($P < 0.05$ for both).

Discussion

To the best of our knowledge, the present study is the first to report that AP cells from ruminants express AMHR2 and that

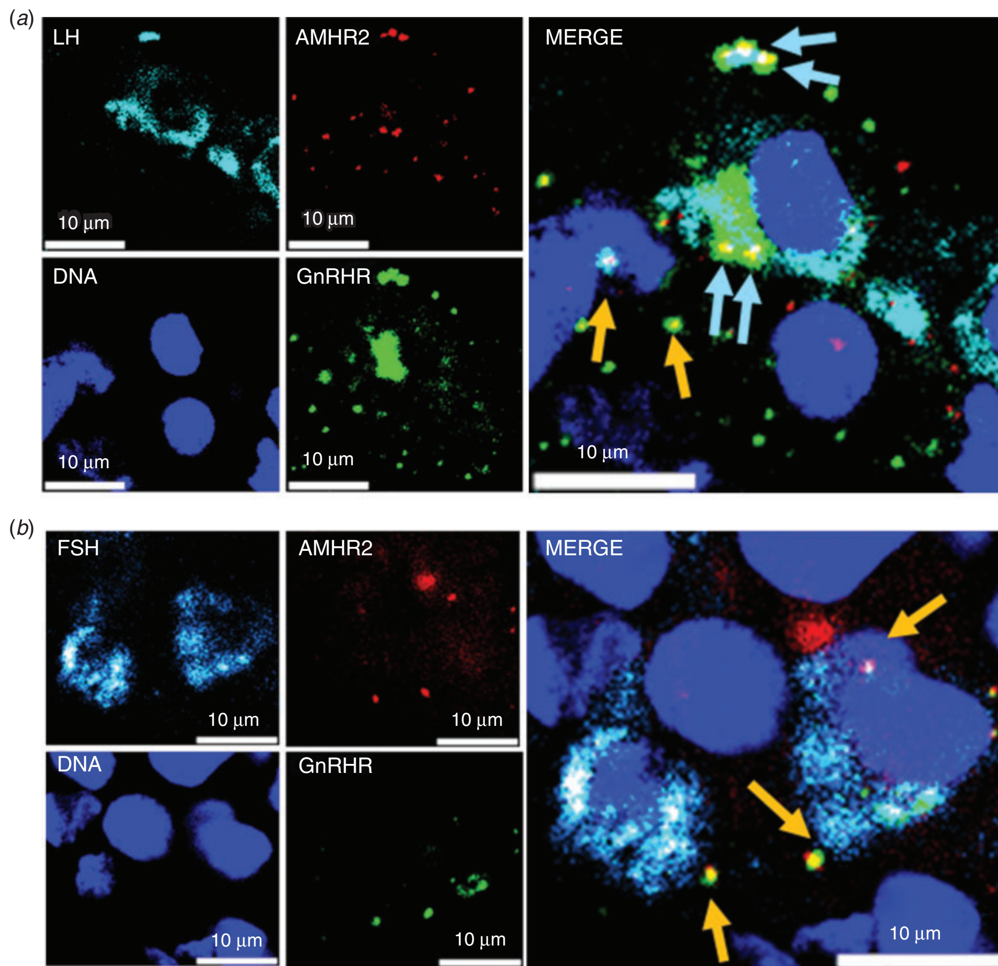

Fig. 4. Triple-fluorescence immunohistochemistry of anterior pituitary (AP) tissue of postpubertal heifers for anti-Müllerian hormone receptor type 2 (AMHR2), gonadotrophin-releasing hormone receptor (GnRHR) and either (*a*) LH or (*b*) FSH. Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (green) and LH or FSH (light blue) with counterstaining by 4′,6′-diamidino-2-phenylindole (DAPI; dark blue). Yellow indicates the colocalisation of AMHR2 and GnRHR on the surface of LH-positive cells (blue arrows) 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 the best focus for the nucleus and cytoplasmic LH. Therefore, these images were taken using the best focus for the membrane receptors while using strong laser power and strong charge-coupled device sensitivity for DAPI and cytoplasmic LH.

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

In the present study, treatment with 10–1000 pg mL^{-1} AMH stimulated FSH secretion in the absence of GnRH. This finding is in agreement with *in vivo* experiments on rats, where AMH stimulates the secretion and expression of FSH (Garrel *et al.* 2016). These data suggest that AMH may bind with AMHR2 to increase FSH secretion from gonadotrophs in ruminants as well. Garrel *et al.* (2016) recently reported that AMH increases both FSHβ expression and phosphorylates SMAD1/5/8 in LβT2 cells, but such increases are blocked by GnRH. In the present study, 1–10 pg mL^{-1} AMH did not change GnRH-stimulated FSH secretion; however, 100–1000 pg mL^{-1} 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 SMAD1/5/8 pathways have important roles.

Multiparous (third parity or higher) Japanese Black cows have significantly higher blood AMH concentrations (100 pg mL^{-1}) than primiparous cows (1–10 pg mL^{-1}) throughout the postpartum period (Koizumi and Kadokawa 2017). In addition the number of days from parturition to first ovulation postpartum is higher in multiparous than

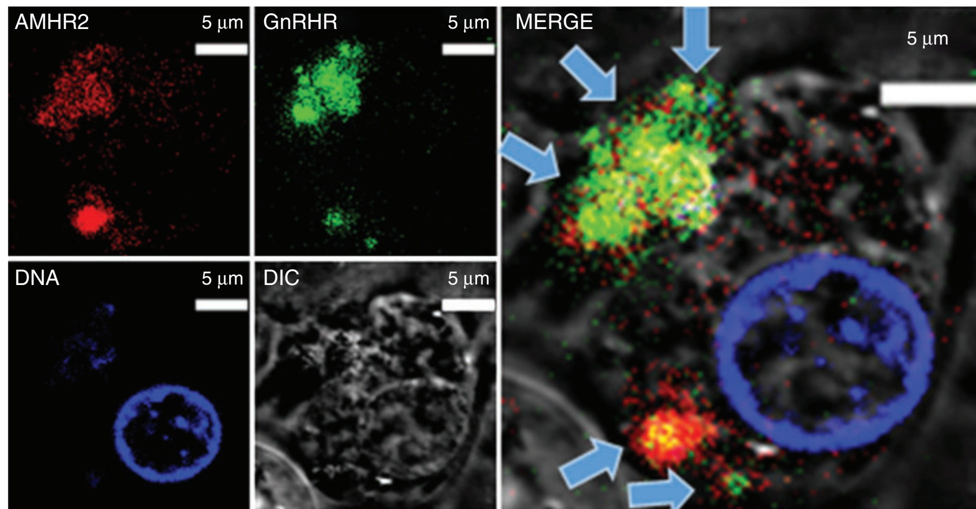

Fig. 5. Fluorescence immunocytochemistry was used to confirm the colocalisation (yellow in the merge panel) of anti-Müllerian hormone receptor type 2 (AMHR2) and gonadotrophin-releasing hormone receptor (GnRHR) on the surface of cultured anterior pituitary (AP) cells (prepared by the CellCover method; see text for details) of postpubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (red), GnRHR (green), DNA (dark blue) and differential interference contrast (DIC) on cultured AP cells that were not treated with Triton X-100 for antibody penetration. Thus, the antibody could only bind AMHR2 and GnRHR on the surface of gonadotrophs. The blue arrows indicate the colocalisation of aggregated GnRHR and aggregated AMHR2.

primiparous Japanese Black cows (Koizumi and Kadokawa 2017). Therefore, the suppressive effect of 100–1000 pg mL^{-1} of AMH on GnRH-stimulated FSH secretion may have an important role in the follicular growth and delayed first ovulation postpartum in multiparous cows.

Intraperitoneal injection with AMH increases FSH concentrations in blood collected 18 h later, but only in prepubertal female rats (Garrel *et al.* 2016). In contrast, the present study showed a significant effect of AMH on FSH secretion from the AP of postpubertal heifers *in vitro*. Therefore, further studies are required to clarify whether there are any differences in AMH effects on FSH secretion among species.

Unlike the hypothalamus, the pituitary gland is located outside the blood–brain barrier (Nussey and Whitehead 2001); therefore, the AMHR2 on gonadotrophs may bind AMH secreted from preantral and small antral follicles. The findings of the present study suggest that AMH, like other members of the TGFβ family, such as inhibin and activin (Kushnir *et al.* 2017), can affect FSH secretion from gonadotrophs. However, little is known about the changes that occur in blood AMH concentrations during the oestrous cycle in ruminants (Pfeiffer *et al.* 2014; Koizumi and Kadokawa 2017). The blood AMH concentration is affected by age and parity (Koizumi and Kadokawa 2017); however, the concentration may not show considerable changes during the oestrous cycle in ruminants *in vivo* (Pfeiffer *et al.* 2014; Koizumi and Kadokawa 2017). Therefore, we must be cautious before concluding that AMH makes a considerable contribution to the control of LH and FSH secretion from gonadotrophs *in vivo*.

The results of the present study suggest that preantral and small antral follicles may control gonadotrophin secretion from the AP in postpubertal heifers. Conversely, FSH suppresses AMH secretion from bovine granulosa cells (Rico *et al.* 2011). Therefore, there may be feedback mechanisms between gonadotrophs and granulosa cells in preantral and small antral follicles. AMH locally decreases the sensitivity of FSH in follicles in many species, including the mouse and sheep (Durlinger *et al.* 2001; Campbell *et al.* 2012; Visser and Themmen 2014). Recently, Ilha *et al.* (2016) reported that *AMH* mRNA levels decrease in both dominant and subordinate follicles during follicular deviation in cows. Thus, both dominant and subordinate follicles become more sensitive to FSH and can be recruited to enter the pool of follicles that may then become dominant (Visser and Themmen 2014). Therefore, AMH may have an important role in both the ovary and gonadotrophs during follicular selection in monovulatory species.

Gonadotrophs are a heterogeneous cell population comprising LH and FSH monohormonal and bihormonal subsets in rats, equines and bovines (Townsend *et al.* 2004; Pals *et al.* 2008; Kadokawa *et al.* 2014). In the present study, fluorescent immunohistochemistry showed AMHR2 expression in LHβ-positive cells as well as FSHβ-positive cells. Furthermore, 100 and 1000 pg mL^{-1} AMH only weakly stimulated LH secretion. Therefore, AMH may control also LH secretion, but weakly. Intraperitoneal injection with AMH increases FSH concentrations in the blood collected 18 h later in rats; however, AMH injection does not significantly increase LH concentrations in the same blood samples (Garrel *et al.* 2016). Therefore, the effect of AMH on LH secretion *in vivo* may not become significant.

It is well known that G-protein-coupled receptor (GPCR) can form functionally active homomers and heteromers with different receptors (Ritter and Hall 2009). We found a strong positive overlap coefficient between AMHR2 and GnRHR on the cell surface. This overlap coefficient was greater than that reported

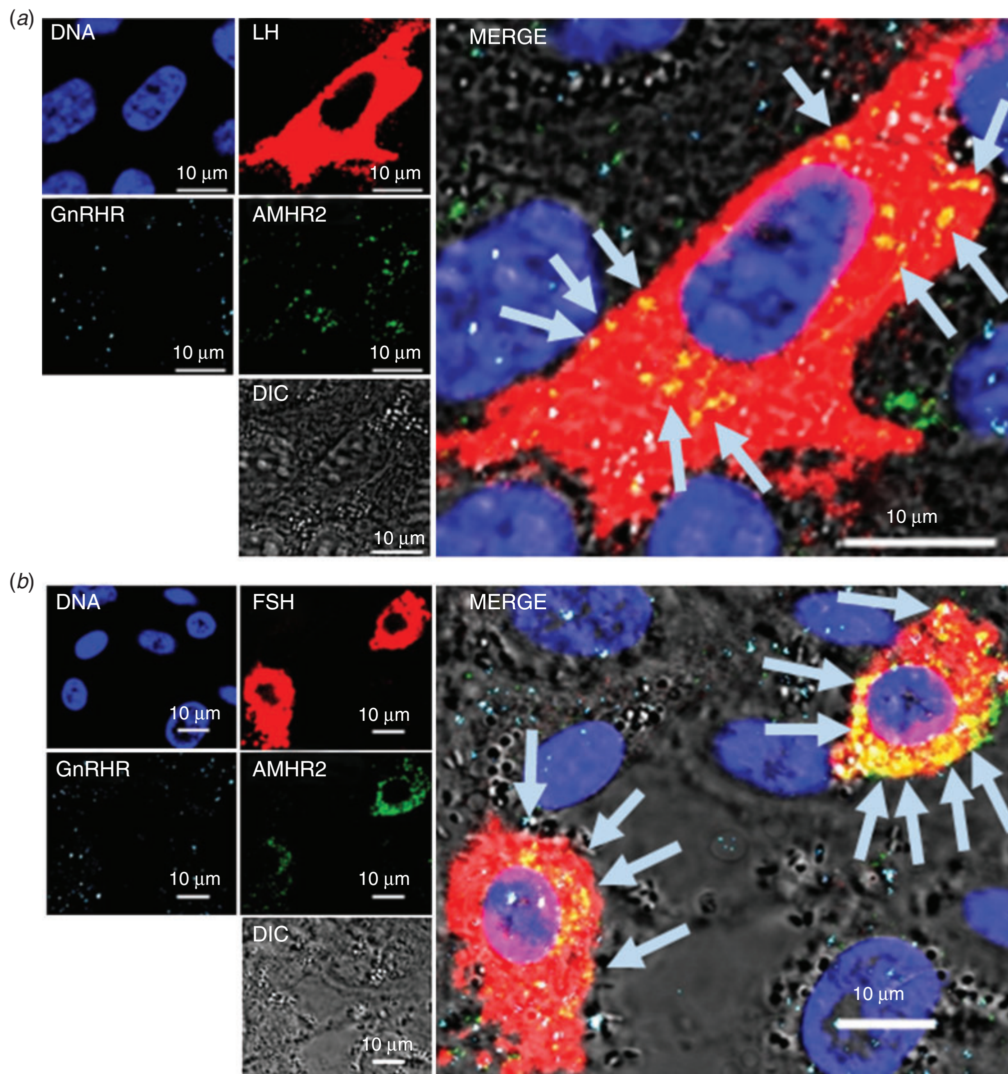

Fig. 6. Triple-fluorescence immunocytochemistry of cultured anterior pituitary (AP) cells (prepared by the paraformaldehyde (PFA)-Triton method; see text for details) of postpubertal heifers for anti-Müllerian hormone receptor type 2 (AMHR2), gonadotrophin-releasing hormone receptor (GnRHR) and either (*a*) LH or (*b*) FSH. Images were captured by laser confocal microscopy for AMHR2 (green), GnRHR (light blue) and LH or FSH (red) with counterstaining by 4′,6′-diamidino-2-phenylindole (DAPI; dark blue). Yellow (arrows) indicates the colocalisation of AMHR2 and LH of FSH in (*a*) LH- and (*b*) FSH-positive cells. These images were taken using the best focus for the membrane receptors while using strong laser power and strong charge-coupled device 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.

between GnRHR and flotillin-1 in cultured LβT2 cells (0.50; Wehmeyer *et al.* 2014) and similar to that we found previously between GnRHR and GPR61 (0.71; Pandey *et al.* 2017) and GPR153 (0.75; Pandey *et al.* 2018) in bovine gonadotrophs. Heterodimerisation among paralogues of GnRHRs of a protochordate results in the modulation of ligand-binding affinity, signal transduction and internalisation (Satake *et al.* 2013). Thus, it is possible that AMHR2 forms a heteromer, affecting ligand-binding affinity, signal transduction and internalisation of GnRHR, and thus the synthesis and secretion of LH and FSH in the AP of vertebrates. Furthermore, a recent study (Hossain *et al.* 2016) suggested that GPR61 forms heteromers with other GPCRs. Therefore, further studies are required to clarify whether GnRHR forms heteromers with GPR61, GPR153 and AMHR2.

In the present study we observed multiple, not single, bands of AMHR2 in western blotting analysis, which has been reported previously. For example, Faure *et al.* (1996) reported three bands (82, 73 and 63 kDa) of dimers, full-length monomers and cleaved monomers. Hirschhorn *et al.* (2015) reported more bands (∼58, ∼69 and ∼71 kDa) of dimers, full-length monomers and cleaved monomers. AMHR2 is present as dimers, full-length monomers and cleaved monomers in bovine ovaries and APs. Treatment with *N*-glycosidase F showed a further two bands (68 and 61 kDa) by cutting down by

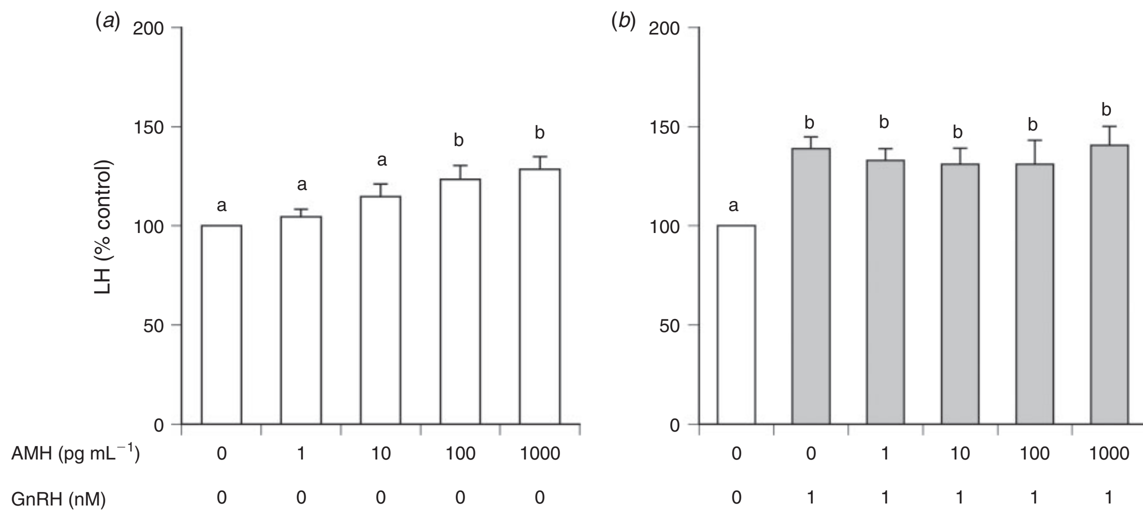

Fig. 7. Comparison of the effects of different concentrations of anti-Müllerian hormone (AMH) in the medium in the (*a*) absence or (*b*) presence of 1 nM gonadotrophin-releasing hormone (GnRH) on LH secretion from cultured anterior pituitary (AP) cells of postpubertal heifers. The LH concentrations in 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. Data are expressed as the mean ± s.e.m. Different letters indicate significant differences ($P < 0.05$).

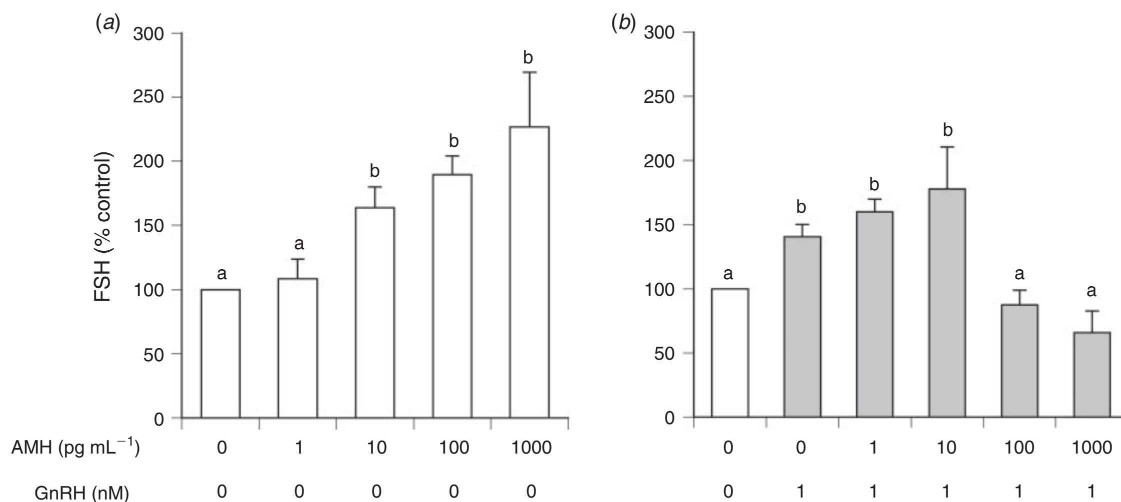

Fig. 8. Comparison of the effects of various concentrations of anti-Müllerian hormone (AMH) in the medium in the (*a*) absence or (*b*) presence of 1 nM gonadotrophin-releasing hormone (GnRH) on FSH secretion from cultured anterior pituitary (AP) cells of postpubertal heifers. The FSH concentrations in 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. Data are expressed as the mean ± s.e.m. Different letters indicate significant differences ($P < 0.05$).

approximately 5 and 2 kDa, because AMHR2 is *O*-glycosylated (Faure *et al.* 1996). The full-length monomers in APs appeared as a doublet, whereas those in the ovary appeared as a single band in the present study. Therefore, the present study suggests that bovine AMHR2 is glycosylated, and the difference in the number of full-length monomers between the AP and ovary may be because of differences in glycosylation.

The anti-AMHR2 antibody revealed similar bands in the two tissues in western blotting analyses. However, the intensity of the bands was weaker for AP than ovarian tissue. Nevertheless, β-actin bands showed weaker staining in AP tissue than ovarian tissue. This suggests that the AP cell lanes were loaded with a lower amount of proteins than expected. A second difference between AP and ovarian cells was the absence of the dimeric AMHR2 band in AP cells. However, this may 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 per lane).

We found that approximately 20% of AMHR2-positive cells were non-gonadotrophs. At the time of manuscript preparation,

no reports had been 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 postpubertal heifers to control gonadotrophin secretion.

Conflicts of interest

The authors declare no conflicts of interest.

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