Reproductive Biology 18 (2018) 432–439

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Production and validation of a polyclonal serum against bovine FSH receptor

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ARTICLE INFO

Keywords:
Polyclonal serum
FSH receptor
Bovine

ABSTRACT

In ovarian granulosa cells, follicle-stimulating hormone (FSH) regulates the proliferation and differentiation events required for follicular growth and oocyte maturation. FSH actions are mediated exclusively through the FSH receptor (FSHR). In cattle, the FSHR gene expression pattern during folliculogenesis and the implications of this receptor in reproductive disorders have been extensively studied. However, the limited availability of specific antibodies against bovine FSHR has restricted FSHR protein analysis. In the present study, we developed an anti-FSHR polyclonal serum by using a 14-kDa peptide conjugated to maltose binding protein. The antiserum obtained was characterized by western blot of protein extracts from bovine follicles, BGC-1 cells and primary cultures of granulosa cells stimulated with testosterone. Also, the blocking effect of serum on estradiol secretion and cell viability after gonadotropin stimulus was characterized in a functional *in vitro* assay. A 76-kDa protein, consistent with the predicted molecular size of full-length FSHR, was detected in ovarian tissue. Besides, two immunoreactive bands of 60-kDa and 30-kDa (only in cultured cells) were detected. These bands would be related to some of the isoforms of the receptor. Therefore, immunohistochemical assays allowed detecting FSHR in the cytoplasm of granulosa cells and an increase in its expression as follicles progressed from primordial to large preantral follicles. These results suggest that the anti-FSHR serum here developed has good reactivity and specificity against the native FSHR. Therefore, this antiserum may serve as a valuable tool for future studies of the biological function of FSHR in physiological conditions as well as of the molecular mechanism and functional involvement of FSHR in reproductive disorders.

1. Introduction

Follicle-stimulating hormone (FSH) belongs to the pituitary glycoprotein hormone family, which also comprises luteinizing hormone, chorionic gonadotropin and thyroid-stimulating hormone. The activity of these hormones is directed through specific cell surface receptors, whose expression determines their site of action [1]. Since FSH acts exclusively through the FSH receptor (FSHR), mechanisms controlling the expression of the receptor determine the FSH-responsive cell population and influence its sensitivity to FSH [2]. FSHR is highly tissue specific, targeting Sertoli and granulosa cells in the male and female gonads, respectively [3]. In ovarian granulosa cells, temporal changes in FSH signaling regulate a number of transcriptional, metabolic, and hormonal activities that are important for the proliferation and differentiation events required for follicular growth and oocyte maturation [2].

FSHR is a glycoprotein that belongs to the superfamily of G protein–coupled receptors. The intracellular actions of these cell surface receptors are mediated through the activation of one or more guanine-nucleotide-binding signal-transducing proteins (G proteins). The receptors of this superfamily consist of a single polypeptide chain of variable length that contains seven transmembrane segments connected by three extracellular loops and three intracellular loops called “serpentine domain”. In particular, FSHR is characterized by the presence of a large N-terminal extracellular domain [4,5]. While the serpentine domains of individual glycoprotein hormone receptors are functionally

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https://doi.org/10.1016/j.repbio.2018.09.001
Received 24 April 2018; Received in revised form 18 June 2018; Accepted 5 September 2018
Available online 13 September 2018

interchangeable and display high sequence identity (~70%), the ectodomains are less similar (~40%) and are responsible for the recognition and binding specificity of hormone [1]. The full-length FSHR mRNA contains 10 exons, nine of which encode the extracellular domain and the other of which encodes both the transmembrane region and the signal transduction region, and is imperative for functionality [6].

In cattle, the FSHR expression pattern during folliculogenesis as well as implications of this receptor in reproductive disorders have been extensively studied from the point of view of gene expression [7–11]. However, the limited availability of specific antibodies against bovine FSHR that guarantee reliable results has restricted FSHR protein analysis.

Polyclonal antibodies are particularly valuable as biological reagents for the study of proteins, cells and tissues and can be used in a broad range of techniques such as ELISA, immunofluorescence and western blot. The production of polyclonal antibodies depends largely on the immunogenicity, quality, quantity and purity of the protein used as antigen [12]. In this sense, the complexity of the FSHR molecule itself represents a challenge for its production as recombinant protein that will then be used as the immunogen mentioned above. On the other hand, the homology between gonadotropin receptors turns the development of specific antibodies against FSHR more difficult [4]. However, the production of some G protein-coupled receptors has been successfully achieved in a number of different homologous and heterologous expression systems as *Escherichia coli*, insect expression systems and yeast expression systems [13].

Based on the fact that the availability of the well-defined anti-FSHR antibodies would thus allow better understanding the biological effects of FSHR, in the present study, we work in the production and characterization of an anti-FSHR polyclonal serum. Thus, we describe the expression of a recombinant FSHR fragment, the generation of a polyclonal serum, and the characterization of its properties by western blot and immunohistochemical assays. Our interest in the study of bovine FSHR is due to its potential importance in reproductive diseases and as a possible therapeutic target.

2. Materials and methods

2.1. Ethical aspects

All the procedures were carried out according to the "Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching" (2010) and were approved by the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina.

2.2. Collection and preparation of tissues

Ovaries from non-pregnant Holstein cows without macroscopic abnormalities in the reproductive system and with follicular development and absence of corpora lutea in the two ovaries, so as to take the samples at the end of the follicular phase, were obtained from a local abattoir. The complete pairs of ovaries (n = 20) were collected within 20 min of slaughter. For protein and/or RNA extraction techniques, these ovaries were refrigerated and immediately transported to the laboratory. The antral follicles were removed using scissors and scalpel dissection. Before the ovaries were dissected, the follicular diameter was measured using calipers and the follicular fluid was aspirated with a syringe and centrifuged at 2000 × *g* for 10 min. The granulosa cell pellets were processed for RNA or protein extraction. Tissues enriched in theca cells were excised from the follicle and processed for protein extraction. Moreover, the ovaries for immunohistochemical assays were fixed in 4% neutral buffered formalin at room temperature (RT) for 8–12 h and processed as detailed below. On the other hand, ovaries for primary cultures were transported to the laboratory in saline at 35 °C and processed as described later.

2.3. Total RNA extraction and cloning of a cDNA fragment of FSHR

Total RNA was isolated from granulosa cells of bovine ovaries (n = 5) using Trizol LS reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions but with slight modifications. Briefly, granulosa cells from small antral follicles (< 5 mm) [14] were homogenized in 750 μL of Trizol reagent and incubated for 5 min on ice. This follicular category was used because, in a previous study, we showed by relative real-time RT-PCR analysis that the expression of FSHR mRNA in granulosa cells was highest in small antral follicles and then decreased significantly as follicles increased in size [10].

The homogenized samples were mixed with chloroform, shaken vigorously and incubated for 15 min at 4 °C. After centrifugation at 12,000 × *g*, the aqueous phase was transferred to a clean tube and the RNA was precipitated by mixing with isopropyl alcohol. The samples were incubated overnight at −20 °C and centrifuged at 12,000 × *g*. RNA pellets were washed once with 75% ethanol and then allowed to dry. RNA was dissolved in diethylpyrocarbonate (DEPC)-water pre-warmed at 55–60 °C. Finally, the purified RNA was assessed spectrophotometrically for quality and quantity by using a SPECTROstarNano Version 2.10 (BMG-LABTECH, Ortenberg, Germany), aliquoted and stored at −80 °C until used. cDNA was synthesized from DNAse I-treated RNA by reverse transcription using random hexamers and Moloney Murine Leukemia Virus enzyme (both from Invitrogen), as described previously [10].

A 320-bp DNA fragment of bovine FSHR (GenBank accession nº NM_174061) was amplified by PCR using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific Company, Espoo, Finland) and the specific primers Bv-fshr4-F (5′-GCTTTCTAGACTGGAAAAATTTGTCAC-3′) and Bv-fshr2-R (5′-CCATGTCGACT*TAA*CATGGATTAAATG-3′). The underlined nucleotides represent the *XbaI* and *SalI* restriction sites, respectively. These restriction sites were included to facilitate the subsequent cloning procedure. The nucleotides in italics indicate a stop codon. The PCR product, named *fshr4*, was cloned into the pGEM-T Easy Vector (Promega, WI, USA). The Fshr4-pGEMT cloning vector was transformed into competent *E. coli* DH5α cells by the heat-shock method [15]. After amplification, this plasmid was isolated and digested by both the *XbaI* and *SalI* restriction enzymes (Promega), according to the manufacturer's instructions. The digestion products were separated on a 1.5% agarose gel and the *fshr4* insert was extracted using Wizard® SV Gel and PCR Clean-Up System (Promega). The *fshr4* insert obtained was subsequently cloned into the pMAL-c2X expression vector (New England Biolabs, MA, USA) containing a *malE* gene which encodes maltose binding protein (MBP) as a carrier protein for further purification. The plasmid pMAL-fshr4 was isolated from the bacterial cells and used to transform the competent *E. coli* strain BL21(DE3) cells to express the recombinant protein. The cloned fragment was checked by sequencing, using the Macrogen Sequencing Service (Macrogen, Korea), confirming a 100% homology with the bovine sequences selected (NM_174061).

2.4. Expression of the recombinant MBP-FSHR4 fusion protein

E. coli strain BL21(DE3) cells were transformed with the pMAL-fshr4 plasmid and selected on Luria-Bertani (LB) solid medium containing ampicillin (100 μg/mL). The transformants were inoculated into 2 mL of 0.5% glucose-supplemented LB medium in test tubes and grown overnight at 37 °C with constant agitation (200 rpm). Then, overnight cultures were transferred into 0.5 L fresh medium in 2-L flasks. When the cultures reached logarithmic growth phase (at OD_{600} of 0.4–0.6), expression of the recombinant MBP-FSHR4 fusion protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG, Promega) with further growth at 30 °C for 4 h. After induction, the cells were harvested by centrifugation at 4000 × *g* for 10 min at 4 °C and

resuspended in ice-cold column buffer (AB; 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT). Next, 2 µL of lysozyme (50 µg/mL) (Thermo Scientific, IL, USA) and 2 µL of DNase I (2500 units/mL) (Invitrogen) were added per 1 mL of column buffer. The resuspended cell solution was lysed by sonication in ice water bath (cycle time: 2 min, pulse: 30%, power: 40%; Sonic Ruptor 250, Omni International, GA, USA) until the sample was clear. The soluble and insoluble fractions were prepared by centrifugation at 12,000 × *g* for 20 min at 4 °C. For each sample, the supernatants (soluble fraction) were collected, and the same volume of column buffer was used to resuspend the pellets (insoluble fraction). Total, soluble and insoluble fractions were analyzed by 12% SDS-PAGE to examine the presence of the MBP-FSHR4 fusion protein. The uninduced control culture and the vector control culture were analyzed in parallel.

To optimize the production of the recombinant protein, culture conditions for expression were optimized in terms of different temperatures (25, 30 and 37 °C), concentrations of IPTG (0.5 and 1.0 mM), and induction period (2–4 h, and overnight). Protein expression was assessed by SDS-PAGE, as described above.

2.5. Amylase affinity purification of the MBP-FSHR4 fusion protein

Cell lysate from 1 L of induced culture was prepared as explained above and the soluble fraction mixed with an amylose resin (New England Biolabs) equilibrated with column buffer AB. Protein binding to the resin was performed in batch for 2 h at 4 °C. After loading the column, the flow-through was collected at a rate of 0.5 mL/min and the column was washed with the same buffer. Proteins bound to the column were then eluted with elution buffer (column buffer with 10 mM maltose). Fractions of 500 µL were collected during elution, and after SDS-PAGE analysis, fractions containing the purified recombinant protein were pooled.

2.6. SDS-PAGE

To assess the production and purification of MBP-FSHR4, the bacterial cell lysates and the fractions obtained from the purification of MBP-FSHR4 were separated by 12% SDS-PAGE and then analyzed by Coomassie brilliant blue R-250 staining.

2.7. Production of anti-FSHR polyclonal serum

Antiserum against FSHR was raised in New Zealand white rabbits. Blood (1.5 mL) was collected prior to immunization to obtain basal serum. This basal serum was used as negative control. Each rabbit was then subcutaneously injected with a mixture of 1 mg of purified MBP-FSHR4 protein mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) on the back and proximal limbs (100 µL/site). Three booster injections were given with 0.5 mg recombinant protein each in incomplete Freund's adjuvant (Sigma-Aldrich) at 3-week intervals. Two weeks after the last injection, blood was collected and kept at 37 °C for 1 h and at 4 °C for 3–4 h to allow blood clotting. The antiserum was collected by centrifugation (3000 × *g* for 10 min), purified using an affinity column (Hi Trap rProtein A FF, Amersham, USA) and stored at –20 °C until further use.

2.8. Primary culture of bovine granulosa cells

The serum-free granulosa cell culture was performed as described by Gutierrez et al. [16], with slight modifications. Briefly, follicles were dissected from the ovaries collected, and those with obvious signs of atresia (avascular theca or debris in the antrum) were discarded. Antral follicles that were ~5 mm in diameter (as measured with calipers, n = 6) were dissected from the ovaries, and follicular fluid was aspirated and centrifuged. The antral cavity was flushed repeatedly with sterile saline buffer (PBS) supplemented with antibiotic-antimycotic 1X (penicillin G sodium 100 IU/mL, streptomycin 100 mg/mL, and amphotericin B as fungizone 25 µg/mL, Gibco by Life Technologies, NY, USA) and granulosa cells recovered by centrifugation at 800 × *g* for 5 min. Granulosa cells were washed three times by centrifugation at 800 × *g* for 5 min each and resuspended in DMEM:F12 (Gibco, MA, USA) supplemented with bovine serum albumin 0.1% (Santa Cruz Biotechnology, TX, USA), FSH 1 ng/mL (Sigma), insulin 0.01 ng/mL (Sigma), and antibiotic-antimycotic 1 × . Cell viability was estimated with 0.4% Trypan blue stain. Cells were seeded into 12-well tissue culture plates at a density of 1×10^5 viable cells per well in 1 mL medium. Cultures were maintained at 37 °C in 5% CO_2 for 2 days before the stimulus and for three days after the stimulus (treatment with testosterone (T), see details below), with half of the medium being replaced every day. At the end of the culture period, cells were collected and stored at –20 °C until total protein extraction for western blot analysis.

2.9. Established bovine granulosa cell line (BCG-1) culture

BGC-1 cells were kindly supplied by Dr. L. Barañao (Instituto de Biología y Medicina Experimental - CONICET, Buenos Aires, Argentina). BGC-1 cells were obtained by spontaneous immortalization of primary cultures [17]. Cells were seeded into 12-well culture plates at a density of 2.5×10^4 viable cells per well in 2 mL of medium and then allowed to attach for at least 4 h before the addition of the stimulus (treatment with T, see details below). Cultures were grown in DMEM:F12 supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotic-antimycotic 1X in the presence of the stimulus. Cells were maintained at 37 °C in 5% CO_2 for 3 days, with half of the medium being replaced every day. Finally, cells were collected and stored at –20 °C until total protein extraction for western blot analysis.

2.10. Treatment of granulosa cells with testosterone

Primary cultures of granulosa cells and BGC-1 cells were treated with 100 ng/mL T (Sigma-Aldrich, St. Louis, MO, USA) for 3 days to induce FSHR expression [18].

2.11. Total protein extraction

The ovarian tissues, primary cultures of granulosa cells, and BGC-1 cells were homogenized in radioimmunoprecipitation assay lysis buffer with a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany), as described previously [19]. Protein concentrations were determined by the Lowry method [20]; Bio-Rad, Hercules, CA, USA).

2.12. Western blotting

The proteins separated by 12% SDS–PAGE were transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl; 0.05% Tween 20, pH 7.5) for 5 h at RT. Rabbit anti-FSHR polyclonal serum (1:5000 dilution) was added to the membranes and shaken overnight at 4 °C. Preimmune serum and polyclonal serum preabsorbed with an excess of the recombinant FSHR4 polypeptide were used as controls. The membranes were then washed five times (5 min each) with TBS-T. Goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, TX, USA) (1: 10,000 dilution) was then added and incubated for 1.5 h at RT. After the membranes were washed, the immunoreactive bands were detected by ECL Prime Western Blotting Detection Reagent according to the manufacturer's instructions (GE Healthcare Life Sciences, Buckinghamshire, UK).

Protein extracts of theca, lymph node, spleen and MAC-T cells (epithelial cell line isolated from bovine mammary tissue [21] stored at −80 °C in our laboratory were used as negative controls.

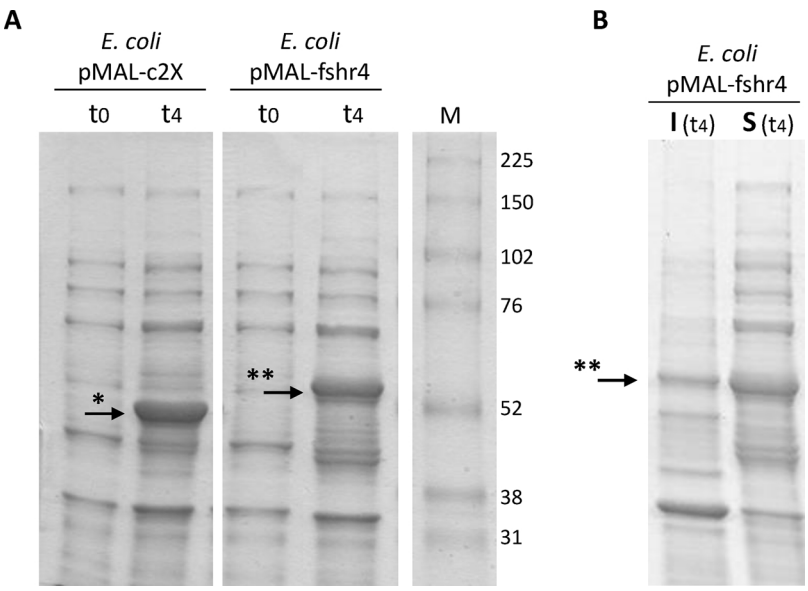

Fig. 1. Expression of the FSHR4 polypeptide of bovine FSHR in *Escherichia coli*. **(A)** Coomassie-stained SDS-PAGE of total cell extracts of *E. coli* BL21 (DE3) carrying pMAL-c2X or pMAL-fshr4 before (t_0) and after induction with IPTG for 4 h (t_4). **(B)** The solubility analysis of the recombinant FSHR4 polypeptide from the soluble (S) and insoluble (I) fractions obtained from an induced *E. coli* (pMAL-fshr4) culture for 4 h (t_4). * MBP protein. ** MBP-FSHR4 fusion protein. M, molecular mass markers.

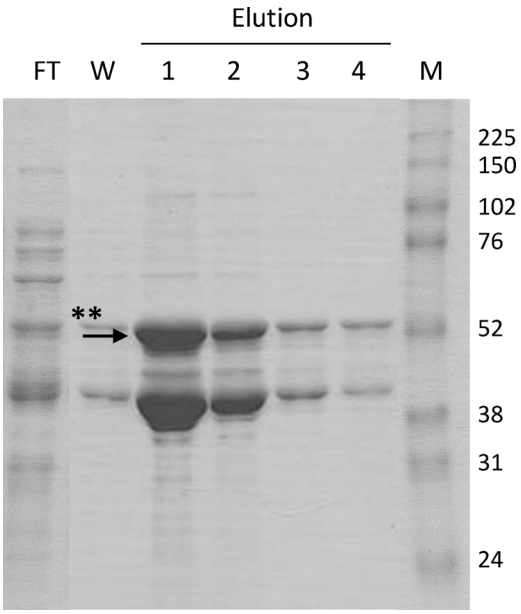

Fig. 2. Affinity purification of the MBP-FSHR4 fusion protein on amylose resin. Elutions were performed with the addition of 10 mM maltose for all fractions (lanes 1–4). ** MBP-FSHR4 fusion protein. FT, flowthrough. W, wash. M, molecular mass markers.

2.13. Immunohistochemical

To locate and quantify FSHR protein expression in the different categories of ovarian follicles, at least five sections of each ovary from 15 cows were processed for indirect immunohistochemistry. A streptavidin-biotin immunoperoxidase method was used for immunohistochemical detection as described previously [22,23]. Briefly, the ovaries fixed in 4% neutral buffered formalin were washed in PBS, dehydrated and embedded in paraffin wax. Sections (4 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and stained with hematoxylin-eosin for a preliminary observation.

After deparaffinization, the antigen was retrieved by incubating the sections in 10 mM sodium citrate solution (pH 6.0) and boiling in a pressure cooker. Endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (v/v) normal goat serum for 20 min at RT. Then, the sections were incubated with the anti-FSHR polyclonal serum at a 1:2500 dilution at 4 °C for 18 h and then with an IgG goat anti-rabbit IgG-B biotin-conjugated secondary antibody (Santa Cruz, Dallas, TX, USA) at a 1:100 dilution for 30 min at RT. The antigens were visualized by the CytoScan™ HRP Detection System (Cell Marque, Rocklin, CA, USA), and 3,3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) was used as chromogen. Finally, the slides were counterstained with Mayer's hematoxylin, dehydrated and mounted. Inactivation of residual endogenous peroxidase activity was confirmed by incubating some sections with DAB alone. The specificity of the secondary antibody was tested with negative control sections processed as described above, except for the replacement of primary antibodies by a non-immune serum (basal serum) [22]. Moreover, the specificity of the secondary antibody was tested by incubation with anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA), a primary antibody against human antigens with a proven negative reaction to cattle tissues. Follicles were classified into primordial, primary, small preantral, large preantral and antral based on pre-established criteria [24].

Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). Images were digitized at × 40 magnification using a Nikon DS-Fi2 (Tokyo, Japan) digital camera mounted on top of a conventional light microscope (Nikon Eclipse Ci-L Ni), as described and validated previously [19,22]. The average immunostaining (% of positive area) was calculated from at least 20 images of the granulosa area in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain) [22].

Also, a cross-reactivity test by immunohistochemistry was carried out in paraffin-embedded tissues archived in our laboratory. Ovarian slides from bitch, sheep, rat and mouse and testicle slides from bulls, pig, mouse, rat and cats were analyzed. Also, a prediction of cross-reactivity was made based on sequence similarity by using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine.

2.14. In vitro functional study of polyclonal anti-FSHR serum

BGC-1 cells cultured and maintained as previously described were seeded into 96-well culture plates at a density of 4×10^3 viable cells per well in 200 μL of medium with 100 ng/mL of T as an estradiol (E2) precursor and to induce FSHR expression. Granulosa cells were cultured

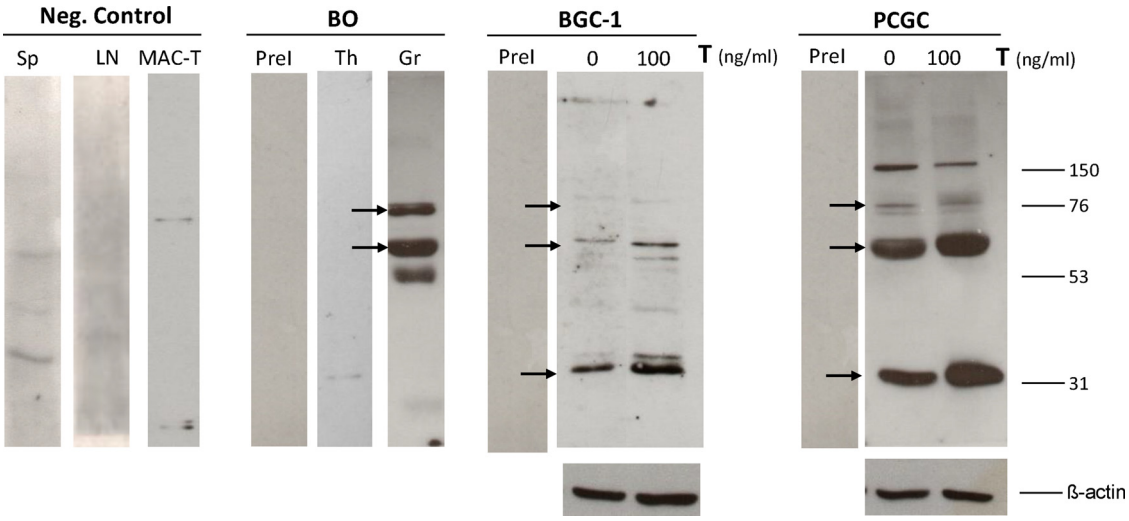

Fig. 3. Western blot analysis of the anti-FSHR polyclonal serum in the ovary and in granulosa cells in culture. Pattern of protein bands detected in theca (Th) and granulosa (Gr) cells from small antral follicles from bovine ovaries (**BO**) and in the **BGC-1** cell line and primary cultures of granulosa cells (**PCGC**) treated with testosterone (T; 100 ng/mL). Preinmune serum (PreI), internal control with β-actin, and negative controls in spleen (Sp), lymph node (LN) and MACT-T cells are shown. Putative bands of FSHR and possible isoforms are indicate (arrows).

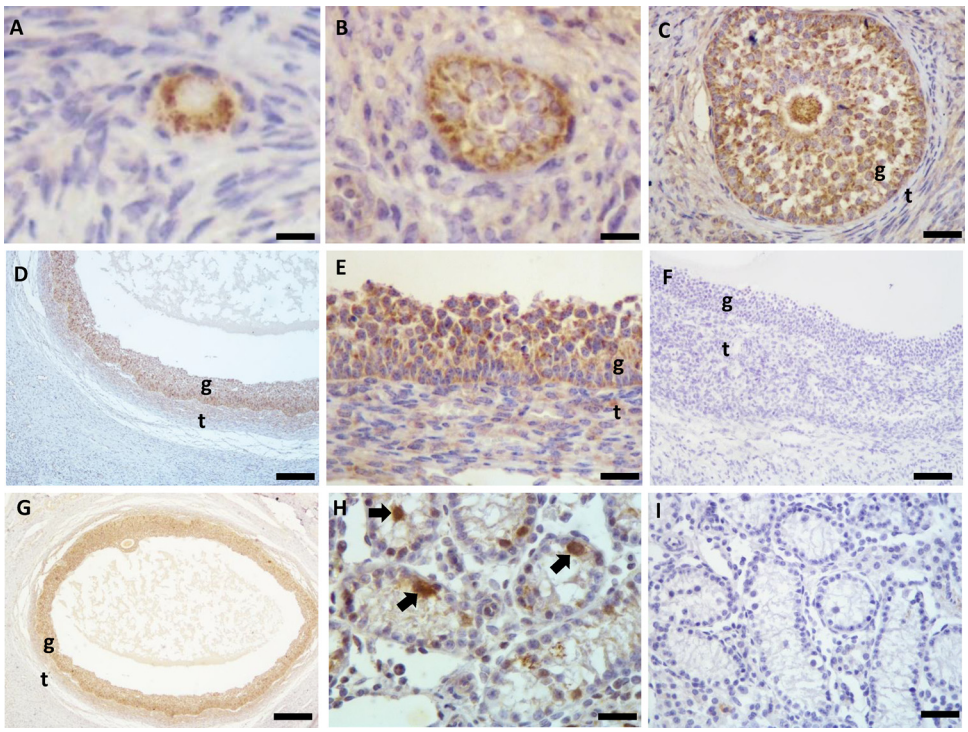

Fig. 4. Representative images of FSHR immunolabeling in (A) primary, (B) small preantral, (C) large preantral, (D,E) and antral follicles from cow. Also the cross reactivity test showed a clear and specific immunostaining in granulosa cells from sheep (G) as well as in Sertoli cells (arrows) from testis bulls (H). Controls with preimmune serum also are shown (F,I). t: theca cells, g: granulosa cells. Bars A & B = 20 µm; C, E, H & I = 40 µm; D & F = 80 µm; G = 100 µm.

with 0.2 UI of equine chorionic gonadotropin (eCG) per well (Biogénesis-Bagó, Argentina) and anti-FSHR polyclonal serum or normal rabbit serum at 1:100, 1:1000 and 1:10,000 dilutions. Also, a control without polyclonal serum or normal serum was analyzed. Fresh medium and hormone and polyclonal serum or normal serum were supplied daily at the appropriate concentration. The daily removal of culture medium allowed dead cells to be removed from the culture wells. Cells were cultured in triplicate wells per treatment and the complete assay was performed in duplicate.

At the end of the culture period (day 3 of culture), the culture medium was stored at - 20 °C and subsequently analyzed, as described below, for E2 production. Cells were washed twice with PBS and exposed to medium with 100 µL of XTT (4x diluted original stock solution (Biotium Inc., CA, USA). After 3 h, the changes in absorbance were recorded by a microplate spectrophotometer SPECTROstarNano at 450 nm with 650 nm of reference wavelength. The absorbance of the medium alone was recorded to determine whether it interfered with the assay.

E2 in culture medium was measured by electrochemiluminescence immunoassay (ECLIA) kits (Roche Diagnostics GmbH, Germany) in a COBAS e411 system (Roche Diagnostics), according to the manufacturer´s instructions, which were previously validated in bovine studies [25]. The assay sensitivity for E2 was 5.00 pg/mL.

2.15. Statistics

The adequate number of images per follicle and the number of follicles per category were confirmed from a sample size calculation

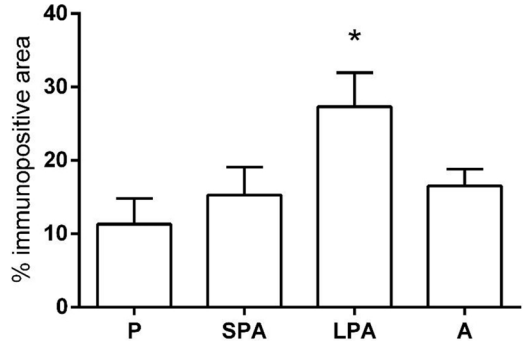

Fig. 5. Relative protein expression (measured as a percentage of immunopositive area) of FSHR in granulosa cells. P: primary; SPA: small preantral; LPA: large preantral and A: antral. Different letters indicate significant differences. Values represent the mean ± SD.

that evaluated the number of samples necessary to obtain an estimate of the immunoexpression that would fall within 0.4 units of the real value. The statistical software package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. The distribution of data was tested for normality using the Kolmogorov–Smirnov test. The percentage of immunopositive area was evaluated by analysis of variance (ANOVA) followed by Duncan's multiple range tests to determine differences between groups. Cell viability and E2 concentration were evaluated by paired t test, analyzing the effect of anti-FSHR polyclonal serum or normal rabbit serum. For all statistical analyses, $p < 0.05$ was considered significant. Results are expressed as mean ± standard deviation (SD).

3. Results

3.1. Heterologous expression of the FSHR4 polypeptide of the bovine FSHR in E. Coli

The bovine *fshr4* cDNA, which spans portions of exons 9 and 10 of full-length FSHR, was successfully amplified by RT-PCR and cloned into the pMAL-c2X expression vector. The derived recombinant plasmid pMAL-fshr4 was introduced into the host *E. coli* BL21(DE3). After evaluating different conditions, the induction of MBP-tagged *fshr4* expression was carried out at 30 °C for 4 h by the addition of 0.5 m M IPTG. Thus, a distinct band with a molecular weight of about 56 kDa, corresponding to the expected size of the MBP-FSHR4 fusion protein, was observed in an induced culture as compared to the a culture without induction. In the case of the empty pMAL-c2X vector, we observed a band of 42 kDa corresponding to the MBP protein alone (Fig. 1A). Therefore, the difference of about 14 kDa was attributed to the FSHR4 polypeptide. Furthermore, according to the SDS-PAGE analysis of the soluble fraction and cell debris pellet, the majority of the induced protein was found in the soluble fraction (Fig. 1B).

3.2. Purification of the MBP-FSHR4 fusion protein

The MBP-FSHR4 protein was purified by amylase affinity chromatography as described above. The SDS-PAGE analysis showed a band corresponding to the MBP-FSHR4 fusion protein (56 kDa) and the presence of another protein band probably corresponding to an MBP-sized breakdown product (molecular mass of 42 kDa) (Fig. 2). The MBP-FSHR4 protein was used to prepare a polyclonal serum.

3.3. Characterization of the immunological specificity of the anti-FSHR polyclonal serum by western blot and immunohistochemistry

Western blot assays of total protein extracts from ovarian tissues, primary cultures of granulosa cells and BGC-1 cells were used to characterize the immunoreactivity and specificity of the anti-FSHR polyclonal serum.

In granulosa cells of small antral follicles, the anti-FSHR polyclonal serum revealed the presence of a ~76-kDa protein, consistent with the predicted molecular size of full-length FSHR. In addition, a ~60-kDa immunoreactive band, which would correspond to some of the isoforms of the receptor, was also detected (Fig. 3). The band of lower molecular mass is a breakdown product. On the other hand, in the BGC-1 cell line and in primary cultures of granulosa cells, a different protein band pattern was observed. The 76-kDa band was accompanied by two other predominant bands of 60 kDa (similar to that found in tissue) and 30 kDa (which could correspond to a new splicing variant generated under *in vitro* culture conditions). In contrast, no positive signal was observed in total protein extracts of theca, lymph node, spleen neither MAC-T cells (Fig. 3) or when using the preimmune serum or when the polyclonal serum was preabsorbed with the recombinant FSHR4 polypeptide. On the other hand, immunohistochemical assays revealed that anti-FSHR polyclonal serum was able to detect FSHR in the cytoplasm of bovine granulosa cells from all the follicular categories analyzed and that the expression increased with the progress of follicles from primordial follicles (*i.e.* before the appearance of the antrum) to large preantral follicles (*i.e.* the late preantral phase) (Fig. 4). A decrease in the expression of FSHR was observed in antral follicles, with immunostaining levels similar to those of small preantral and primary follicles (Fig. 5). In contrast, no immunostaining was observed in theca cells. These results suggest that the anti-FSHR polyclonal serum has good reactivity and specificity against FSHR in ovarian cells.

The cross reactivity test by immunohistochemistry showed a clear and specific immunostaining in granulosa cells from sheep as well as in Sertoli cells from bulls without staining of Leydig cells (Fig. 5). Different patterns of non-specificity were observed in the other species. Also, the prediction of cross-reactivity made by BLAST showed sequence similarity in *Ovis aries* (99%), *Sus scrofa* (93%), *Equs caballus* (91%), *Homo sapiens* (89%), *Mus musculus* (87%), and *Rattus norvegicus* (87%).

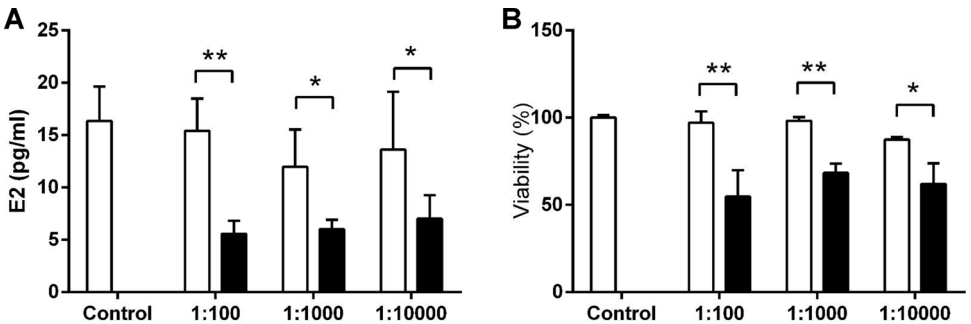

Fig. 6. *In vitro* functional study showing the blocking effect of anti-FSHR polyclonal serum in relation to normal serum on E2 secretion (A) and cell viability (B), (expressed as % of control) after eCG treatment. Values represent means ± SEM. Significance is denoted by asterisks: *$p < 0.05$; **$p < 0.01$.

3.4. Functional characterization of polyclonal anti-FSHR serum

The eCG response of BGC-1 cells *in vitro* on day 3 of culture is shown in Fig. 6. eCG stimulated E2 production of granulosa cells, whereas anti-FSHR polyclonal serum had a blocking effect in relation to normal serum ($p < 0.05$). Also, anti-FSHR polyclonal serum had a significant blocking effect ($p < 0.05$) on cellular activity induced by eCG, as evidenced by the analysis of cell viability by XTT.

4. Discussion

In previous studies, the protein and mRNA expression of gonadotropin receptors in bovine ovarian follicles has been analyzed by a variety of methods such as *in situ* hybridization, semi-quantitative RT-PCR, and real-time quantitative RT-PCR [7,8,18,26–30]. However, no studies have systematically evaluated the protein expression of FSHR in cattle, probably due to the lack of availability of specific antibodies for this species. Only a few reports in cattle have described immunoblotting, but using antibodies raised against human receptor peptides, which showed discrepancies in the results in terms of the molecular mass found. In both bovine granulosa and cervical preparations, Mizrachi and Shemesh [31] observed the presence of a 75-kDa protein, which corresponds with the predicted molecular size of FSHR. On the other hand, Nuttinck et al. [32] studied the protein expression of bovine FSHR in cumulus–oocyte complexes in reducing conditions and observed two major 110- and 60-kDa immunoreactive protein species. The authors argued that the 60-kDa band probably corresponded to the molecular mass of bovine FSHR whereas and that the nature of the 110-kDa band was unknown.

In this study, a portion of the bovine FSHR gene (826–1145 bp) coding for a 14-kDa polypeptide (FSHR4) was successfully expressed in the *E. coli* prokaryotic expression system as a fusion to the MBP tag. The different parameters tested to optimize the expression levels of FSHR4 showed that they were highest under the conditions of 30 °C with 0.4 mM IPTG for 4 h. *E. coli* is one of the earliest and most widely used hosts for the production of heterologous proteins [33] due to a number of factors, including its relatively inexpensive cost, ease of manipulation and rapid growth rate. However, as a bacterial system, *E. coli* has limitations to express more complex proteins due to the lack of sophisticated machinery to perform post-translational modifications, resulting in poor solubility of the protein of interest, which is produced as an inclusion body [34]. Costa et al. [35] made a thorough description of different fusion tags that can be added to the target protein to increase its solubility and/or ease its purification from cell cultures. The most popular fusion tags are MBP, N-utilization substance protein-A, thioredoxin, glutathione S-transferase, ubiquitin and SUMO. The reasons why these fusion tags act as solubility enhancers remain unclear. In the case of MBP, it has been shown to possess an intrinsic chaperone activity [36]. In the present study, to establish an efficient approach to purify the FSHR4 polypeptide, MBP purification was applied, with the MBP-tag showing the advantages of increased solubility and mild elution conditions.

Our polyclonal serum against FSHR characterized herein was able to detect a ~76-kDa band in granulosa cells from bovine ovarian follicles, consistent with the size of full-length FSHR. In addition, a ~60-kDa and a ~30-kDa (only in *in vitro* assays) immunoreactive bands were also detected, which would correspond to the different receptor isoforms previously described [37]. The bovine FSH-receptor cDNA was cloned and sequenced for the first time in 1994 by Houde et al. [6], who described that it is 2085 bp in length and consists of 10 exons. Subsequently, a number of different alternatively spliced mRNA isoforms for bovine FSHR have been identified [37]. There are two other exon 10 containing isoforms apparent in bovine granulosa cells, one of which lacks exon 9 and the other of which lacks exons 4, 5, and 9 [37]. Moreover, it is possible that other alternative transcripts may exist in cattle since any transcript lacking exon 10 would not have been amplified in the PCR experiments described by these authors. However, the functions of these receptor variants remain unclarified. Also, it is not even known if any of alternatively spliced mRNAs are translated *in vivo* in bovine. However, it has been shown that these isoforms participate differentially in hormone binding and signaling mechanisms during different phases of reproduction in the ewe [38]. Besides, the ability of anti-FSHR polyclonal serum to block the stimulus by eCG demonstrates that this segment of the receptor would actively participate in the response mechanisms to hormonal stimuli. The FSHR4 fragment spanning the terminal region of the extracellular domain of the receptor near the first transmembrane segment would be functional and necessary for the response to the ligand. Finally, our immunohistochemical analyses suggest that the polyclonal serum raised against the MBP-FSHR4 recombinant fusion protein was able to recognize the native FSHR in cattle and sheep.

In conclusion, our results provide evidence that the FSHR4 fragment expressed in *E. coli* prokaryotic host cells had a correct conformation and appropriate antigenic integrity to produce antibodies with high specificity to the native FSHR in ovarian tissue. Therefore, this antiserum may serve as a valuable tool for future studies on the biological function of FSHR in physiological conditions as well as on the molecular mechanism and functional involvement of FSHR in reproductive disorders.

Acknowledgments

This study was supported by a grant from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 2012-2638 & PICT 2016-1790) and Universidad Nacional del Litoral (CAI + D – 2011 & 2016 Programs). We are grateful to the staff of the Laboratorio de Biología Celular y Molecular Aplicada (ICIVET-Litoral UNL CONICET) for their technical support during processing of the samples.

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