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## CHEMERIN (RARRES2) Decreases In Vitro Granulosa Cell Steroidogenesis and Blocks Oocyte Meiotic Progression in Bovine Species<sup>1</sup>

**Running Title:** CHEMERIN OR RARRES2 IN BOVINE FOLLICLES

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### ABSTRACT

CHEMERIN or RARRES2 is a new adipokine that is involved in the regulation of adipogenesis, energy metabolism and inflammation. Recent data suggest that it also plays a role in reproductive function in rats and humans. Here we studied the expression of CHEMERIN and its three receptors (CMKLR1, GPR1 and CCRL2) in the bovine ovary and investigated the in vitro effects of this hormone on granulosa cell steroidogenesis and oocyte maturation. By RT-PCR, immunoblotting and immunohistochemistry, we found CHEMERIN, CMKLR1, GPR1 and CCRL2 in various ovarian cells, including granulosa and theca cells, corpus luteum, and oocytes. In cultured bovine granulosa cells, INSULIN, IGF1 and two insulin sensitizers, metformin and rosiglitazone increased *rarres2* mRNA expression whereas they decreased *cmklr1*, *gpr1* and *cclr2* mRNA expression. Furthermore, TNF alpha and ADIPONECTIN significantly increased *rarres2* and *cmklr1* expression, respectively. In cultured bovine granulosa cells, human recombinant CHEMERIN (hRec, 200 ng/ml) reduced production of both progesterone and estradiol, cholesterol content, STAR abundance, CYP19A1 and HMGCR proteins, and the phosphorylation levels of MAPK3/MAPK1 in the presence or absence of FSH ( $10^{-8}$  M) and IGF1 ( $10^{-8}$  M). All these effects were abolished by using an anti-CMKLR1 antibody. In bovine cumulus-oocyte complexes, the addition of hRec (200 ng/ml) in the maturation medium arrested most oocytes at the GV stage and this was associated with a decrease in MAPK3/1 phosphorylation in both oocytes and cumulus cells. Thus, in cultured bovine granulosa cells, hRec decreases steroidogenesis, cholesterol synthesis and MAPK3/1 phosphorylation probably through CMKLR1. Moreover, in cumulus-oocyte complexes, it blocked meiotic progression at the germinal vesicle stage and inhibited MAPK3/1 phosphorylation in both the oocytes and cumulus cells during in vitro maturation.

**Summary Sentence:** The chemerin adipokine is produced by ovarian follicular cells, it decreases in vitro bovine granulosa cell steroidogenesis and de novo cholesterol synthesis and arrests at germinal vesicle stage bovine oocyte during in vitro maturation.

**Keywords:** adipokine, signaling pathways, ovary, cholesterol, reproduction, granulosa

## INTRODUCTION

CHEMERIN (16kDa), also called retinoic acid receptor responder protein 2 (RARRES2) or tarazotene-induced gene 2 (TIG2), is an adipokine mainly expressed by white adipocytes but also by macrophage, plasmacytoid dendritic cells and natural killer cells in rodents and humans [1, 2]. It is produced and released as an inactive precursor called prochemerin that is quickly converted to its active form by proteolytic cleavage of its C-terminus [3]. Three G protein-coupled receptors are able to bind chemerin with high affinity, namely CMKLR1 (chemokine receptor-like 1 or ChemR23, [4]), GPR1 (C protein-coupled receptor1, [5]) and CCRL2 (C-C chemokine receptor-like 2, [6]). CHEMERIN binding to CMKLR1 triggers calcium mobilization, receptor and ligand internalization, and cell migration [4]. In contrast to CMKLR1, very few studies have investigated signal transduction pathways coupled to GPR1 in response to CHEMERIN. CHEMERIN binding to CCRL2 does not induce signaling pathways or ligand internalization but could control CHEMERIN bioavailability and improve the interaction between CHEMERIN and CMKLR1 on adjacent cells [3, 7]. CHEMERIN is involved in the regulation of immunity, adipogenesis, lipolysis and glucose metabolism [8-10]. Plasma CHEMERIN levels are associated with body mass index and plasma triglycerides [10]. They are high in obese patients and in those with diabetes [8, 10]. Plasma CHEMERIN levels are also increased in patients with polycystic ovary syndrome (PCOS) [11]. CHEMERIN regulates insulin sensitivity in rodents and humans. Furthermore, in sheep, a recent study shows that a CHEMERIN analog in vivo regulates INSULIN secretion related to glucose metabolism and the release of triglycerides [12].

Recently, some data have suggested that CHEMERIN could affect female and male reproductive functions. Indeed, CHEMERIN is present in the ovary and testis in rodents and humans [13-15] but also in the rat placenta and in human umbilical cord blood [16]. In rats, elevated CHEMERIN levels suppress follicle development [17]. Furthermore, serum CHEMERIN levels fall as pregnancy progresses, suggesting a regulatory role of CHEMERIN in maternal-fetal metabolism [18]. In rat testes, CHEMERIN and its three receptors are developmentally regulated and highly expressed in Leydig cells [15]. Moreover, *in vitro* treatment with CHEMERIN suppressed the human chorionic gonadotropin (hCG)-induced testosterone production from primary Leydig cells [15]. Our laboratory has recently shown that CHEMERIN and CMKLR1 are present in the human ovary, especially in granulosa cells (GC) and in the human granulosa cell line KGN [14]. In primary human granulosa cells we observed that CHEMERIN decreases IGF1-induced steroidogenesis and cell proliferation through a decrease in the activation of IGF1R signalling pathways [14]. In a 5 $\alpha$ -dihydrotestosterone-induced rat model, mRNA and protein expression levels of CHEMERIN and CMKLR1 were significantly higher in the ovaries: this could help to explain the induction of antral follicle growth arrest [13, 17]. The same authors have also shown that CHEMERIN decreases FSH-induced steroidogenesis in rat follicle and granulosa cell culture [13, 19]. In patients with polycystic ovary syndrome (PCOS), serum CHEMERIN levels and subcutaneous or omental

adipose tissue were significantly increased [11]. However, treatment with metformin, an insulin sensitizer, for six months, strongly reduced serum CHEMERIN levels in PCOS patients. PCOS is the most common cause of anovulation and infertility, affecting 5% to 10% of women of reproductive age [20]. It is characterized by hyperandrogenism, chronic anovulation, and, occasionally, obesity [20]. In addition to its reproductive consequences, PCOS is a metabolic disorder associated with insulin resistance and hyperinsulinemia [21].

In dairy cows, the decrease in fertility associated with increases in milk yield is related, in part, to the intensity of selection on milk production [22]. Dairy cows have been selected to produce more milk, mostly through their ability to mobilize fat to support milk production. This results in a strong loss of adipose tissue after calving that is associated with alterations in blood metabolite and hormone profile including adipokines such as LEPTIN and ADIPONECTIN [23-25] which in turn could influence fertility. Plasma CHEMERIN during lactation has not yet been determined in dairy cow. However, adipose CHEMERIN mRNA abundance is raised during adipose tissue mobilization in Holstein cows [26], but whether this elevated CHEMERIN affects ovarian follicular function in cattle is unknown. In the present study, we identified CHEMERIN and its receptors in the bovine ovary and investigated the effects of human recombinant CHEMERIN (hRec) in cultured granulosa cell steroidogenesis and during in vitro oocyte maturation.

## **MATERIALS AND METHODS**

### *Ethics*

All procedures were approved by the Agricultural Agency and the Scientific Research Agency, and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

### *Hormones and Reagents*

Recombinant human IGF1 and INSULIN and metformin were obtained from Sigma (St Louis, MO). Purified ovine FSH (lot no.AFP-7028D, 4453 IU/mg, FSH activity=175 x activity of (oFSH-S1) used for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD. Recombinant human CHEMERIN and TNF $\alpha$  were purchased from R&D (Lille, France). Recombinant human LEPTIN was obtained from Biovendor (Euromedex, Souffelweyersheim, France). Recombinant bovine ADIPONECTIN and RESISTIN were obtained from Clinisciences (Nanterre, France). Rosiglitazone was a gift from B. Staels (Lille, France). Thymidine methyl-H3 was obtained from Amersham Life Science, (Arlington Heights, IL).

### *Antibodies*

Rabbit polyclonal antibodies to PRKAA1 and HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) were purchased from Upstate Biotechnology Inc (Lake Placid, NY). Rabbit polyclonal antibodies to phospho-MAPK3/1 (Thr202/Tyr204), phospho-MAPK14 (Thr180/Tyr182), phospho-AKT1 (Ser 473), AKT1 and phospho-PRKAA1 Thr172 were obtained from New England Biolabs Inc (Beverly, MA). Mouse monoclonal antibodies to VCL and CYP19A1 were obtained from Sigma (St. Louis, MO) and Serotec (Varilhes, France), respectively. Rabbit polyclonal antibodies against CYP11A1, STAR, and HSD3B were generously provided by Dr. Dale Buchanan Hales (University of Illinois, Chicago, IL) and Dr.

Van Luu-The (CHUL Research Center and Laval University, Quebec, Canada), respectively. Rabbit polyclonal antibodies to MAPK3 (C14), MAPK14 (C20), CHEMERIN, GPR1, CCRL2 and CMKLR1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies were used at 1:1,000 dilution in Western blotting.

#### *Collection of Bovine Tissue and Primary Culture of Bovine Granulosa Cells*

Bovine tissues (corpus luteum, ovarian cortex, small and large follicles and granulosa cells) were recovered from a local slaughterhouse. Tissue and cell samples for mRNA and protein extraction were frozen in liquid nitrogen and stored at -80°C. Bovine ovaries were obtained from adult cows collected in a slaughterhouse and transported aseptically before dissection. Then, granulosa cells (GC) were recovered from small antral follicles (3 to 5 mm) in modified McCoy 5A medium followed by 5 min centrifugation. Cells were washed, counted in a hemocytometer and cultured in McCoy 5A supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l), 0.1% BSA, 5 mg/l transferrin, 20 mg/l selenium, and 10% fetal bovine serum (FBS, PAA laboratories, les Mureaux, France). Approximately  $4 \times 10^5$  viable cells were seeded in each plastic multiwell containing 1 ml medium. After 24h of culture, cells were starved with McCoy 5A medium containing 1% of FBS for one night and then incubated in fresh culture medium with or without test reagent for the appropriate time. All cultures were performed in a water-saturated atmosphere of 95% air/5% CO<sub>2</sub> at 37 °C.

#### *RNA Isolation and RT-PCR*

As described previously [14], total RNA was extracted by using Trizol reagent according to the manufacturer's procedure (Invitrogen). Reverse transcription (RT) and polymerase chain reaction (PCR) were then carried out. Briefly, 1 µg of total RNA was reverse transcribed for 1h at 37°C in 20 µl final volume of reaction containing 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM MgCl<sub>2</sub>, 200 µM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ), 50 pmol of oligo(dT) 15, 5 U of ribonuclease inhibitor, and 15 U of MMLV reverse transcriptase. 2 µM of each set of specific primers as described in Table 1 were used. PCR conditions were DNA denaturation at 95°C for 5 min, 95°C for 1 min, 58°C for 1 min and 72°C min for 35 cycles before a final extension at 72°C for 10 min. For this, 2 µl of RT reaction mixture were added in 25 µl final volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U of Taq polymerase. Results were viewed on 1.5% agarose gel stained with ethidium bromide and the amplified DNA was extracted and sequenced by the Genome Express company (Meylan, France). Consumables for RT-PCR were obtained from Sigma (l'Isle d'Abeau Chesnes, France), and Moloney Murine Leukemia Virus reverse transcriptase and RNase inhibitor were from Promega, Madison, WI).

#### *Real Time Polymerase Chain Reaction (PCR)*

After the reverse transcription, the bovine cDNAs of granulosa cells were diluted 1:5. The real time PCR was made in 20 µl final volume containing 10 µL iQ SYBR Green supermix (Bio-Rad), 0,25 µL of each primer (10 µM), 4,5 µL of water and 5 µl of template. The cDNA templates were amplified and detected with the MYIQ Cyclor real time PCR system (Bio-Rad) with the following protocol: 1 cycle for 5 min at 95°C to denature the sample and then 40 cycles, 1 min at 95°C for denaturation, 1 min at 60°C for hybridization, 1 min at 72°C for stretching and finally 1 cycle for 5 min at 72°C for final elongation. We used as control gene for normalization

the *actb* (F 5'-ACGGAACCACAGTTTATCATC-3' and R 5'-GTCCCAGTCTTCAACTATAACC3') *rpl19* (F 5'-AATCGCCAATGCCAACTC-3' and R 5'-CCCTTTCGCTTACCTATAACC-3') and *ppia* (F 5'-GCATACAGGTCCTGGCATCT-3' and R 5'-TGTCCACAGTCAGCAATGGT-3'). We determined the primer efficiency, by diluting the pool of cDNA of different samples. The efficiency was between 1.7 and 2. We detected the amplification signal with a fluorophore reporter, SYBR Green. Then we determined the cycle threshold (Ct) to quantify the sample, all samples being used in duplicate. The cycle threshold represents the minimal number of cycles necessary to ensure that the fluorescence emitted by SYBR green exceeds the detection threshold. The logarithms of Ct obtained were calculated and the linear regression equation of these points allowed the efficiency of each primer pair to be calculated ( $E=10^{-1/k-1}$ , where K= slope). This Ct value is compared with a housekeeping gene, and the ratio indicates the adipokine expression, the values being obtained by subtracting the Ct value of chemerin from that of the reference gene.

#### *Protein Extraction and Western Blot*

Primary bovine granulosa cells and all other tissues or cells were homogenized as previously described [27]. Lysates were incubated on ice for 30 min before centrifugation at 12,000 x g for 20 min at 4°C. The pellet was eliminated and the samples were stored at - 80°C. The protein concentration for each condition was measured using a BCA protein assay. Samples were denatured and submitted to electrophoresis on 12 % SDS-polyacrylamide gel at 90 volts before being transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilland, France). Then the membranes were blocked for 30 min with TBS-Tween-milk 5 % and incubated with specific primary antibodies (dilution 1/1000) for 16 h at 4°C. After several washes, membranes were incubated for 1h 30 min with the secondary antibodies conjugated with HRP anti-rabbit or anti-mouse IgG at 1/5000 final dilution. Proteins were revealed by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using a G:Box SynGene (Ozyme) with the GenSnap software (release 7.09.17). Quantification was performed with the GeneTools software (release 4.01.02).

#### *Cholesterol Content*

The cholesterol content was quantified with the Cholesterol E-test assay kit (Wako). Standard solutions were prepared from 0 to 50 mg/mL. Four microliters of each sample and of the standard solution were loaded onto a 96-well plate, followed by the addition of 300 µL of reaction mixture in each well. The absorbance was recorded using a spectrophotometer (wavelength, 600 nm). The cholesterol concentrations were corrected according to the protein contents of the samples measured by a BCA protein assay.

#### *Immunohistochemistry*

Bovine ovary sections were deparaffinised, hydrated and microwaved in antigen unmasking solution for 5 min before being allowed to cool to room temperature. Sections were then washed in PBS for 5 min, and immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). Ovary sections were washed for 5 min in PBS followed by incubation for 20 min in PBS with 5% lamb serum in order to eliminate non-specific background labeling. Sections were incubated overnight at 4°C with PBS containing rabbit primary antibody raised against either CHEMERIN (RARRES2), CMKLR1, GPR1 or CCRL2 at a dilution of 1:100. Sections were washed twice for

5 min each time in a PBS bath, followed by 30 min incubation at room temperature with a “ready to use” labelled Polymer-HRP anti-rabbit (DakoCytomation Envision Plus HRP system, Dako, Ely, UK). Finally sections were washed twice in PBS and the staining was revealed by incubation at room temperature with 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB+Substrate Chromogen System, DakoCytomation). We used primary antibodies with rabbit IgG as negative controls.

#### *Thymidine Incorporation into Granulosa Cells*

Primary bovine granulosa cells were cultured for 24h in McCoy 5A medium and 10 % FBS (Fetal Bovine Serum). Cells were plated in a 24-well plate ( $2 \times 10^5$  viable cells/well) and four replicates were tested for each experimental condition (CHEMERIN, IGF1...) for each culture. After several washes and one night serum starvation, cells were cultured for 24 h with 1  $\mu\text{Ci}/\mu\text{l}$  of [3H] thymidine (Amersham Life Science, Arlington Heights, IL) in the presence or absence of chemerin and/or IGF1 ( $10^{-8}$  M) and or FSH ( $10^{-8}$  M). After that, thymidine was removed with PBS and fixed with cold 50% trichloroacetic acid for 15 min on ice. Finally, cells were lysed by 0.5 N NaOH and the radioactivity was counted in a  $\beta$ -photomultiplier by adding scintillation fluid (Packard Bioscience).

#### *Progesterone and Estradiol Radio-immunoassay*

Progesterone and estradiol concentrations were measured in serum free medium from primary bovine granulosa cells after 48h of culture by a radioimmunoassay protocol as previously described [27]. Cells were plated in a 48-well plate ( $10^5$  viable cells/well) and we tested eight replicates for each experimental condition (CHEMERIN, IGF1...) for each culture. The results were expressed as the concentration of steroid (ng/ml)/ protein concentration/well. The intra- and inter-assay coefficients of variation for progesterone were less than 10 % and 11 %, respectively. The intra- and inter-assay coefficients of variation for estradiol were less than 7% and 9%, respectively. Results are means  $\pm$  SEM and are representative of six to eight independent cultures with each condition in quadruplicate.

#### *Bovine Oocyte Collection and In Vitro Maturation*

Bovine ovaries were collected from a slaughterhouse in sterile NaCl solution and maintained at 37°C until aspiration. The cumulus-oocyte complexes (COCs) were aspirated from follicles 3-8 mm in diameter using an 18-G needle connected to a sterile test tube and to a vacuum line (100 mmHg) as previously described [28]. COCs were then selected under a dissecting microscope. Expanded or non-intact COCs were eliminated: only intact COCs were washed in TCM Hepes 199 (Sigma) supplemented with BSA (0.4%) and gentamycine (2.5ml/L) under mineral oil (Sigma). The COCs were cultured in TCM 199 (Sigma) with BSA (4mg/ml) supplemented or not with IGF1 ( $10^{-8}$ M) and/or chemerin (200 ng/ml) for 10 or 22 h at 39°C in 5% CO<sub>2</sub> in air with saturated humidity. Each oocyte group contained at least 25 oocytes. After maturation COCs were denuded by pipetting with 0.5% hyaluronidase (Sigma) and the DNA colored with Hoechst before mounting.

#### *Statistical Analysis*

All experimental results are presented as the mean  $\pm$  SEM. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) or a t-test to test differences. If ANOVA revealed significant effects, it was followed by the Student Newman Keuls test.

## RESULTS

### *CHEMERIN and its Receptors (CMKLR1, GPR1 and CCRL2) mRNA and Protein Expression in the Bovine Ovary*

RT-PCR analysis with RNA from dissected corpus luteum (CL), ovarian cortex (Cx), small (SF) and large follicles (LF) and fresh granulosa cells from SF (GC SF) and LF (GC LF) resulted in the amplification of four cDNAs corresponding to fragments of *chemerin* (*rarres2*) (266 bp), *cmklr1* (400 bp), *gpr1* (571 bp) and *ccrl2* (131 bp) (Figure 1A). By quantitative RT-PCR we show that *rarres2* mRNA is more highly expressed in granulosa cells from SF than those from LF (Figure 1B). Similar results were obtained by using two other reference genes, *rpl19*, and *ppia*, (Supplemental Figure S1, and all other supplemental data, is available online at [www.biolreprod.org](http://www.biolreprod.org)). Only the ratios with *actb* are shown in Figure 1B. In contrast, chemerin receptors (*cmklr1*, *gpr1* and *ccrl2*) mRNA expression were similarly expressed in all the ovarian compartments or cells studied (data not shown). Immunoblotting of protein extracts revealed one band corresponding to the CHEMERIN (16 kDa), CMKLR1 (43 kDa), GPR1 (41 kDa) and CCRL2 (40 kDa), showing that CHEMERIN and its receptors are produced in the bovine ovary (Figure 1C). Immunohistochemistry with ovarian sections from bovine follicles confirmed the immunoblot findings and revealed CHEMERIN and its three receptors in oocytes and theca cells (Figure 1D, Supplemental Figure S2). Thus, CHEMERIN (RARRES2) and CHEMERIN receptors are present in the different bovine ovarian follicular cells.

### *Effect of INSULIN, IGF1, Metformin and Rosiglitazone on rarres2 and its Three Receptors mRNA Expression in Bovine Granulosa Cells*

We next investigated the effect of INSULIN ( $10^{-8}$ M), IGF1 ( $10^{-8}$ M) and two insulin sensitizers, metformin ( $10^{-6}$ M) and rosiglitazone ( $10^{-8}$ M) on *rarres2*, *cmklr1*, *gpr1* and *ccrl2* mRNA expression in primary bovine granulosa cells. Overnight-starved cells (with 1% FBS) were incubated for different times (12h and 24h) with INSULIN ( $10^{-8}$ M), IGF1 ( $10^{-8}$ M), metformin ( $10^{-6}$ M) or rosiglitazone ( $10^{-8}$ M). We have previously shown in human granulosa cells that insulin sensitizers including metformin and rosiglitazone regulate expression of visfatin, another adipokines, after 12 and 24h of stimulation [29]. By real-time quantitative PCR, we showed that after 12h of stimulation, INSULIN, IGF1, metformin and rosiglitazone increased *rarres2* mRNA expression (Figure 2A) whereas they decreased mRNA expression of *cmklr1* (except for rosiglitazone, Figure 2B), *ccrl2* (Figure 2C) and *gpr1* (Figure 2D). Similar results were obtained by using two other reference genes (*rpl19*, and *ppia*). Only the ratios with *actb* are shown in Figure 2. Results were consistent at 24 hours of stimulation (data not shown).

### *Effect of Adipokines RESISTIN, ADIPONECTIN, LEPTIN and TNF $\alpha$ on rarres2, cmklr1, gpr1 and cclr2 mRNA Expression in Bovine Granulosa Cells*

It is well known that bovine or ovine granulosa cells express several adipokines and adipokines receptors including resistin [30], adiponectin and its receptors (ADIPOR1 and ADIPOR2) [31, 32], leptin and its receptor [33], and TNF $\alpha$  and its receptors [34]. Furthermore these adipokines have already been shown to modulate in vitro granulosa cell steroidogenesis. Consequently, we studied the effect of these adipokines on *rarres2*, *cmklr1*, *gpr1* and *ccrl2* mRNA expression in primary bovine granulosa cells. Overnight-starved cells (with 1% FBS) were incubated for 12h with RESISTIN (100 ng/ml), ADIPONECTIN (10



µg/ml), LEPTIN (10 ng/ml) or TNFα (10 ng/ml). By real-time quantitative PCR we observed that after 12h of stimulation, only TNFα increased *rarres2* mRNA expression (Figure 3A) whereas only ADIPONECTIN increased mRNA expression of *cmklr1* (Figure 3B). We observed similar results when cells were stimulated for 24 hours. We detected no effect of the adipokines on the mRNA expression of *gpr1* and *ccrl2* whatever the time of stimulation (data not shown).

#### *Effects of Human Recombinant CHEMERIN (hRec) on Basal and FSH or IGF1-Stimulated Progesterone and Estradiol Productions in Bovine Granulosa Cells*

To investigate the effect of hRec on the production of progesterone and estradiol, bovine granulosa cells were incubated with various concentrations of hRec (0, 12, 25, 50, 100 and 200 ng/ml) for 48 h, or with hRec for 48 h in the presence or absence of FSH ( $10^{-8}$  M) or IGF1 ( $10^{-8}$  M). Secretion of both progesterone (Figure 4A) and estradiol (Figure 4B) were inhibited by hRec treatment from the concentration of 25 ng/ml ( $P < 0.001$ ). In the presence of FSH ( $10^{-8}$  M) or IGF1 ( $10^{-8}$  M), hRec (200 ng/ml, 48 h) decreased progesterone and estradiol secretion by almost twofold ( $P < 0.001$ ) (Figure 4C and D).

#### *Effects of a Neutralizing CMKLR1 Antibody on hRec-Inhibited Production of Progesterone and Estradiol in Bovine Granulosa Cells*

We next examined whether the hRec-induced decrease in the production of progesterone and estradiol was mediated by CMKLR1 receptor. We blocked CHEMERIN/CMKLR1 signaling with an anti-CMKLR1 antibody (CMKLR1 Ab, 1µg/ml) beginning one hour prior to application of hRec or IGF1 or FSH and persisting throughout 48h of stimulation. Rabbit IgG was used as an isotype control for the rabbit anti-human CMKLR1 neutralizing antibody. As shown in Figure 5A and B, the neutralizing antibody CMKLR1 (CMKLR1 Ab) totally abolished the CHEMERIN-induced decrease in the production of progesterone and estradiol in the presence and absence of FSH and IGF1. Incubation with rabbit IgG in the same conditions did not restore these inhibitory effects of hRec (Figure 5 A and B). Thus, hRec reduces progesterone and estradiol secretion at least through CMKLR1 in bovine granulosa cells.

We next determined whether the inhibitory effects of hRec on steroid production were due to effects on the protein levels of three crucial enzymes of steroidogenesis (HSD3B, CYP11A1 and CYP19A1) or that of STAR, a cholesterol carrier. As shown in Figure 6A and B, hRec treatment (200 ng/ml, 48h) reduced by at least two-fold the production of STAR and CYP19A1 proteins, not in the absence (basal state) but in the presence of FSH and IGF1 whereas no effect was observed on the protein levels of HSD3B and CYP11A1 (data not shown). These effects were totally abolished in the presence of the CMKLR1 neutralizing antibody (CMKLR1 Ab, 1µg/ml). Thus, the decrease in steroid secretion in response to hRec may be due to a reduction in the amounts of the CYP19A1 and STAR proteins through activation of CMKLR1.

#### *Effects of Human Recombinant CHEMERIN (hRec) on Cholesterol Content and HMGCR Protein Level in Bovine Granulosa Cells*

We next investigated the effect of hRec on the IGF1- or FSH-induced cholesterol content in granulosa cells in the absence or in the presence of the neutralizing CMKLR1 antibody for 48 hours (same conditions used as those for steroidogenesis). As shown in Figure 7A, hRec (200 ng/ml) halved the cholesterol content and the protein level of HMGCR in granulosa cells in the absence or the presence of FSH ( $10^{-8}$ M) or IGF1 ( $10^{-8}$ M). Incubation of cells with the neutralizing CMKLR1 antibody for 48 hours abolished these inhibitory effects. Thus, hRec could

decrease steroidogenesis through an inhibition of cholesterol synthesis through CMKLR1 in bovine granulosa cells.

#### *Effects of Human Recombinant CHEMERIN (hRec) on Bovine Granulosa Cell Proliferation and Viability*

We also examined the effect of human recombinant CHEMERIN on the number of bovine granulosa cells in culture, either by induction of mitosis or by altering the cell viability. [<sup>3</sup>H]-Thymidine incorporation by primary bovine granulosa cells treated with hRec (200 ng/ml) was determined after 24 hours of culture in the presence or in the absence of FSH (10<sup>-8</sup> M) or IGF1 (10<sup>-8</sup> M). As expected, FSH and IGF1 treatment significantly increased [<sup>3</sup>H]-thymidine incorporation (data not shown). However, CHEMERIN treatment did not affect cell proliferation in the basal state or in response to IGF1 or FSH (data not shown). As revealed by staining with trypan blue, chemerin treatment (200 ng/ml) had no effect on cell viability in the absence or presence of FSH and IGF1 (data not shown). Thus, hRec decreased steroid production in response to IGF1 or FSH without affecting the proliferation or viability of bovine granulosa cells.

#### *Signaling Pathways Involved in the hRec Effects in Bovine Granulosa Cells*

It is well known that G-protein coupled receptors (GPCR) activate the MAPK3/MAPK1 signaling pathway that is involved in the regulation of steroidogenesis [35]. As shown in Figure 8A, hRec treatment significantly inhibited phosphorylation of MAPK3/MAPK1 after 10 and 30 min of stimulation in bovine granulosa cells. Under the same conditions, phosphorylation of AKT1, MAPK14 and PRKA was unchanged (data not shown). Incubation of cells with the CMKLR1 neutralizing antibody (CMKLR1 Ab, 1 μg/ml) totally abolished the hRec-induced decrease of MAPK3/MAPK1 phosphorylation in the absence or in the presence of FSH (10<sup>-8</sup>M, 48h) or IGF1 (10<sup>-8</sup>M, 48h) (Figure 8B). Incubation with rabbit IgG in the same conditions did not restore these inhibitory effects of hRec (Figure 8B). Thus, hRec inhibits MAPK3/MAPK1 phosphorylation through CMKLR1 in bovine granulosa cells.

#### *Effects of hRec Treatment on the Nuclear Maturation of Bovine Oocytes in COCs and Progesterone Secretion of Bovine COCs During IVM*

We also studied the effects of hRec (200 ng/ml) on the meiotic progression of bovine oocytes in COCs during IVM. For the control group, after 10h of IVM, about 40% of oocytes underwent GVBD (Figure 9B). After 22h of culture in IVM medium, most oocytes underwent GVBD (Figure 9B), and about 85% of oocytes had progressed to the metaphase II stage, and less than 10% remained at the GV stage (Figure 9B). Conversely if COCs matured in the presence of 200ng/ml of CHEMERIN, meiotic progression was inhibited (Figure 9B). Furthermore, if COCs matured for 22h in IVM medium supplemented with hRec (100, 200 or 400 ng/ml), 30 to 60% of oocytes remained at the GV stage, (Figure 9B,C). Thus, hRec treatment of COCs during IVM resulted in meiotic arrest in a dose-dependent manner. Progesterone secretion by cumulus cells is known to play a key role in bovine oocyte maturation [36, 37]. We therefore investigated the effects of CHEMERIN treatment on progesterone secretion by COCs. The addition of hRec to the maturation medium for 22 h significantly decreased progesterone secretion in COCs (Figure 10A).

### *Effects of hRec Treatment on MAPK3/1 Phosphorylation Levels in Bovine Oocytes and Cumulus Cells in COCs after IVM*

We investigated the molecular mechanisms involved in the effects of hRec on the nuclear maturation of bovine oocytes in COCs by determining the levels of MAPK3/1 phosphorylation in the presence or absence of hRec (200 ng/ml) in COCs allowed to mature in vitro for 22 h. As shown in Figure 10B and C, the level of MAPK3/1 phosphorylation increased in the oocyte and in cumulus cells from COCs during IVM. The addition of hRec (200 ng/ml) to the maturation medium for 22h decreased MAPK3/1 phosphorylation by a factor of two in oocytes and by a factor of four in cumulus cells from COCs. Thus, hRec treatment during IVM decreased MAPK3/1 phosphorylation in COCs.

## **DISCUSSION**

In this study, we demonstrated for the first time that CHEMERIN (RARRES2) and its three receptors CMKLR1, GPR1 and CCRL2 are present in the bovine ovarian follicle. In bovine cultured granulosa cells, INSULIN, IGF1 and two insulin sensitizers (metformin and rosiglitazone) increased *rarres2* expression whereas they decreased mRNA expression of *cmklr1*, *gpr1* and *ccrl2*. In addition, we showed that hRec decreased basal and IGF1 or FSH-induced steroidogenesis, probably through CMKLR1, in cultured bovine granulosa cells. This was associated with a reduction in the cholesterol content and in the levels of STAR, CYP19A1, HMGCR and MAPK3/1 phosphorylation. Furthermore, we have observed for the first time that the addition of hRec (200 ng/ml) to the maturation medium arrested most of the bovine oocytes at the GV stage in COCs suggesting that CHEMERIN could regulate not only granulosa cell steroidogenesis but also nuclear maturation in bovine oocytes during IVM.

We found CHEMERIN and its receptors CMKLR1, GPR1 and CCRL2 at mRNA and protein level in all the different bovine ovarian compartments including corpus luteum, cortex, small and large follicles. In bovine follicles, CHEMERIN and CMKLR1 are present in theca and granulosa cells, cumulus cells and in oocytes. CHEMERIN and CMKLR1 have already been localized in the human and rat ovary [2, 14, 19]. Other adipokines such as LEPTIN [38], ADIPONECTIN [31] and RESISTIN [30] have been previously identified in bovine follicular cells. Using real-time PCR we found that *rarres2* was significantly more expressed in granulosa cells from small follicles than those observed in other follicular cells. The mRNA expression of the three receptors was similar for all follicular cells. In human, CHEMERIN circulates in plasma as different isoforms [39]. Its plasma concentration is about 100-200 ng/ml [40, 41]. In cattle, the plasma CHEMERIN concentrations are not yet known. In our experiments, we stimulated bovine granulosa cells with 200 ng/ml, a concentration close to those observed in human plasma. The DNA sequences of bovine *rarres2* and its receptor, *cmklr1* are highly homologous to those of humans, mice, and pigs [42] suggesting also a high identity for the amino acid sequences. Thus, we used human recombinant CHEMERIN to determine the effects of chemerin on the bovine follicular cells. In our study, we examined the effect of INSULIN, IGF1 and two insulin sensitizers, metformin and rosiglitazone on the mRNA expression of *rarres2* and its three receptors (*cmklr1*, *gpr1* and *ccrl2*) in primary bovine granulosa cells. We showed that INSULIN, IGF1 and both insulin sensitizers increased *rarres2* expression, whereas they decreased mRNA expression of the three CHEMERIN receptors. The stimulatory effect of INSULIN on *rarres2* expression is in good agreement with the literature. Indeed, short-term hyperinsulinaemia upregulates adipocyte CHEMERIN production in vitro and INSULIN induces CHEMERIN release from adipocytes within 24h in mice [43]. Conversely, the stimulatory effect

of metformin on CHEMERIN expression observed in our study is not in agreement with two different studies. Indeed, it has been shown that metformin treatment for six months decreased serum CHEMERIN concentration in women with PCOS [11]. In addition, in the white adipose tissue and serum in high-fat-diet-induced insulin-resistant rats, metformin decreased the expression of CHEMERIN [44]. Thus, these data suggest a tissue and species dependence of the metformin effect on CHEMERIN expression. We also studied the effect of various adipokines (RESISTIN, ADIPONECTIN, LEPTIN and TNF $\alpha$ ) on *rarres2* and *cmklr1* mRNA expression in bovine granulosa cells. Interestingly, we observed that TNF $\alpha$  and ADIPONECTIN significantly increased *rarres2* and *cmklr1* expression, respectively. Such stimulatory effects on *rarres2* and *cmklr1* expression or CHEMERIN plasma levels have been already described in other cell types. For example, at least two studies showed that TNF- $\alpha$  treatment increased bioactive CHEMERIN levels in adipocytes [42, 45] TNF $\alpha$  is a proinflammatory cytokine associated with insulin-resistance and inflammation that is known to inhibit steroidogenesis in bovine granulosa cells [46]. In our study we showed that CHEMERIN strongly inhibited steroid production. Thus, CHEMERIN could be involved in the TNF $\alpha$  effect in bovine granulosa cells. In a good agreement with our results, Wanninger et al., 2012 showed that ADIPONECTIN upregulates *cmklr1* mRNA and protein level in primary human hepatocytes [47]. Thus, our results show that *rarres2* and *cmklr1* expression can be regulated by other adipokines in bovine granulosa cells.

In the present study, we investigated the effect of hRec on bovine granulosa cell steroidogenesis. As expected, we observed that IGF1 and FSH alone increase progesterone and oestradiol secretion by bovine granulosa cells [27]. We showed that hRec significantly inhibited effect steroid production in basal state and in response to IGF1 or FSH and this effect was abolished by using a blocking CMKLR1 antibody. Our results are in good agreement with those described by Wang et al., 2013, that reported that mouse recombinant CHEMERIN decreases FSH-induced steroidogenesis in rat granulosa cells [19]. Furthermore, in previous work in primary human granulosa cells and in the KGN granulosa cell line, we showed that hRec impairs steroid production in response to IGF1 but not FSH, suggesting a species effect of CHEMERIN [14]. During ovarian steroid hormone synthesis, cholesterol is first transported into the mitochondrial inner membrane, facilitated by the steroidogenic acute regulatory protein (STAR), and then converted to the important sex steroid progesterone under sequential actions of the mitochondrial enzyme CYP11A1 and endoplasmic reticulum enzyme HSD3B. Progesterone is then further enzymatically processed into estrogens through the action of various enzymes including CYP19A1. In the present study, hRec decreased the protein levels of STAR and CYP19A1 induced by IGF1 or FSH. These latter results could explain the decrease in progesterone and estradiol secretion. CHEMERIN is known to modulate various signaling pathways including MAPK3/1 and MAPK14 in myoblast and gastric cells [48, 49]. AKT1 and PRKA (AMPK) in muscle [50]. In bovine granulosa cells, we showed that CHEMERIN stimulation in the short term (1 to 30 min) or long term (48h) decreased MAPK3/1 phosphorylation, whereas no significant effect was observed for MAPK14, AKT1 and PRKA phosphorylation. Some studies reported that the MAPK3/1 signaling is a positive regulator in IGF1 or FSH-induced steroid production in cultured rat, human and bovine granulosa cells [27, 35, 51]. In primary human granulosa cells and in rat Leydig cells, CHEMERIN treatment inhibited phosphorylation of MAPK3/1 pathways in response to IGF1 or hCG, respectively [14, 15]. Thus, MAPK3/1 is probably a molecular event involved in the inhibitory effect of CHEMERIN on bovine granulosa cell steroidogenesis.

Steroidogenesis depends on the supply of its precursor, cholesterol, derived from intracellular and extracellular sources. Even if lipoproteins are the major source of cholesterol for steroidogenic cells [52], cellular cholesterol can be derived from the *de novo* synthesis pathway in granulosa cells. Indeed, stimulation of cholesterol biosynthesis plays a fundamental role in FSH action in pig granulosa cells [53]. In bovine granulosa cells, stimulation of HMGCR, a key enzyme controlling *de novo* cholesterol synthesis, is important in progesterone production and cell proliferation in response to IGF1 [54]. In our study, hRec decreased the cholesterol content and the HMGCR reductase protein levels. Taken together, our results suggest that CHEMERIN inhibits steroidogenesis through a decrease of the cholesterol carrier STAR and CYP19A1 amount but also through a reduction in cholesterol *de novo* synthesis in bovine granulosa cells. The inhibitory effects of CHEMERIN on bovine granulosa cell steroid production, cholesterol content and MAPK3/1 phosphorylation in response to IGF1 and FSH were abolished by using an anti-CMKLR1 antibody, which suggests that there is a crosstalk between CHEMERIN/CMKLR1 signaling axis and IGF1R and FSH-R in modulating granulosa cell function. Further investigations are needed to better understand this potential cross-talk. Moreover, it also appears important to inhibit the two other CHEMERIN receptors, GPR1 and CCRL2 in order to determine their role in the granulosa cell functions. In our study, we did not observe any effect of CHEMERIN on cell proliferation and viability in primary bovine granulosa cells, whereas Kim et al, 2012 observed an apoptotic effect of chemerin on granulosa cell in follicle culture of DHT-treated rats and showed that CHEMERIN suppressed FSH and GDF9 stimulated follicular growth [17]. The dose of CHEMERIN used in both studies was quite similar, so we can suggest that the effect of CHEMERIN on cell proliferation and viability depend on the species and also the culture conditions.

In the present study, we showed that hRec induced an arrest at the GV of the bovine oocyte after 22 h of IVM. We also observed that the addition of hRec to the bovine COC's maturation medium strongly reduced progesterone secretion and MAPK3/1 phosphorylation in both oocyte and cumulus cells. Several studies have shown that progesterone stimulates oocyte maturation [36, 37]. Thus, CHEMERIN could block bovine nuclear oocyte maturation through an inhibition of progesterone production by COCs. Furthermore, it is well known that MAPK3/1 activation in oocyte occurs during the first hours of maturation in many species, including cattle. In bovine oocytes, it is associated with the GVBD stage [55]. Thus, the decrease in CHEMERIN-induced MAPK3/1 phosphorylation observed in bovine oocyte and cumulus cells could help to explain the inhibitory effect of CHEMERIN on oocyte nuclear maturation. It will be interesting to determine the involvement of cumulus cells in the CHEMERIN effects during IVM. For this, we are investigating the effects of CHEMERIN on oocyte nuclear maturation when the oocytes are separated from their cumulus cells.

In conclusion, we have demonstrated for the first time the presence of CHEMERIN and its three receptors CMKLR1, GRP1 and CCRL2 in the bovine ovary. In primary bovine granulosa cells, CHEMERIN decreased steroidogenesis and cholesterol synthesis in the basal state and in response to IGF1 or FSH through at least CMKLR1. This was associated with a reduction in protein level of STAR, CYP19A1, HMGCR and phosphorylation of MAPK3/1. Furthermore, we showed that the addition of hRec to bovine COC's maturation medium arrested most of the oocytes at the GV stage and decreased MAPK3/1 phosphorylation in oocytes and cumulus cells. Further investigations are required to determine the *in vivo* effects of CHEMERIN in bovine folliculogenesis.

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## FIGURE LEGENDS

### Figure 1: CHEMERIN (RARRES2) and its receptor CMKLR1, GPR1 and CCRL2 expression in the bovine ovary

**A.** RT-PCR analysis of the mRNAs for *rarres2*, *cmklr1*, *gpr1* and *ccrl2* in corpus luteum (CL), ovarian cortex (Cx), small (SF) and large (LF) follicle, and granulosa cells from SF (GC SF) and LF (GC LF). Tissues or cells from seven different Prim Holstein cows were used.

**B.** Expression of *rarres2* mRNA in the bovine ovarian follicle. mRNA expression of *rarres2* was measured by quantitative real time-polymerase chain reaction in CL, Cx, SF LF, and in GC SF and GC LF. *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. Tissues or cells from seven different Prim Holstein cows were used. Results are represented as mean  $\pm$  S.E.M. Different letters indicate significant differences at  $p < 0.05$ .



C. Detection of CHEMERIN (RARRES2), CMKLR1, GPR1, CCRL2 proteins by immunoblotting in CL, Cx, SF and LF and in granulosa cells from SF (GC SF) and LF (GC LF). VLC is used as a loading control (n = 7).

D. Localization of CHEMERIN and CMKLR1 in the bovine ovary by immunohistochemistry. DAB-immunoperoxidase staining was performed on paraffin-embedded bovine ovary using antibodies against CHEMERIN (1/100), CMKLR1 (1/100) or no primary antibodies but rabbit IgG (1/100). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. CHEMERIN and CMKLR1 are present in all bovine ovarian cells. AT: antrum, Oo: Oocyte, T: Theca cells, GC: Granulosa cells Bars = 100  $\mu$ m. Immunohistochemistry was performed on six different cows.

**Figure 2: Effect of INSULIN, IGF1, metformin and rosiglitazone on *rarres2* (A), *cmklr1* (B), *ccrl2* (C) and *gpr1* (D) mRNA expression in primary bovine granulosa cells**

The mRNA expression of *rarres2*, *cmklr1*, *ccrl2* and *gpr1* was measured by quantitative real-time polymerase chain reaction in primary bovine granulosa cells after 12h of stimulation with or without rosiglitazone (Rosi,  $10^{-8}$ M), metformin ( $10^{-6}$ M), INSULIN ( $10^{-8}$ M) or IGF1 ( $10^{-8}$ M). *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. Results represent six to eight cultures of primary bovine granulosa cells. Results are represented as mean  $\pm$  S.E.M. Different letters indicate significant differences at  $p < 0.05$ .

**Figure 3: Effect of adipokines (RESISTIN, ADIPONECTIN, LEPTIN and TNF $\alpha$ ) on *rarres2* and *cmklr1* mRNA expression in primary bovine granulosa cells**

The mRNA expression of *rarres2* and *cmklr1* was measured by quantitative real-time-polymerase chain reaction in primary bovine granulosa cells after 12h of stimulation with or without RESISTIN (100 ng/ml), ADIPONECTIN (10 $\mu$ g/ml), LEPTIN (10 ng/ml) and TNF $\alpha$  (10 ng/ml). *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. Results represent six to eight cultures of primary bovine granulosa cells. Results are represented as mean  $\pm$  S.E.M. Different letters indicate significant differences at  $p < 0.05$ .

**Figure 4: Effect of CHEMERIN treatment on basal and FSH- or IGF1-stimulated secretion of progesterone and estradiol by bovine granulosa cells**

Granulosa cells from small bovine follicles were cultured for 48h in a medium with serum and then in serum-free medium in the presence or in the absence of various doses of hRec (human recombinant CHEMERIN, A and B) for 48h, or in presence or absence of 200 ng/ml hRec  $\pm$   $10^{-8}$  M FSH or  $\pm$   $10^{-8}$  M IGF1 (C and D) as described in Materials and Methods. The culture medium was then collected and analyzed for progesterone (A, and C) and estradiol (B and D) content by RIA. Results are expressed as ng/ml/protein concentration/well. Results are means  $\pm$  SEM of six independent experiments. Bars with different letters are significantly different ( $P < 0.05$ ).

**Figure 5: Effect of CMKLR1-antibody treatment on basal and FSH- or IGF1-stimulated steroid secretion in response to chemerin in bovine granulosa cells.**

Overnight -starved granulosa cells from small bovine follicles were preincubated with/without 1  $\mu$ g/ml CMKLR1 antibody for 1 h and then treated for 48h with or without 200 ng/ml hRec  $\pm$   $10^{-8}$  M FSH or  $\pm$   $10^{-8}$  M IGF1 as described in Figure 4. The culture medium was then collected and

analyzed for progesterone (A) and estradiol (B) content by RIA. Results are expressed as ng/ml/protein concentration/well. Results are means  $\pm$  SEM of six independent experiments. Bars with different letters are significantly different ( $P < 0.05$ ).

**Figure 6: Effect of CHEMERIN treatment on the amounts of STAR and CYP19A1 proteins and the potential involvement of CMKLR1 in bovine granulosa cells.**

Protein extracts from bovine granulosa cells preincubated for 1 h with or without 1  $\mu$ g/ml CMKLR1 antibody and then cultured for 48h with or without 200 ng/ml hRec  $\pm$   $10^{-8}$  M FSH or  $\pm$   $10^{-8}$  M IGF1 were subjected to SDS-PAGE as described in *Materials and Methods*. The membranes were incubated with antibodies raised against the STAR (A) and CYP19A1 (B) proteins. Equal protein loading was verified by reprobing membrane with an anti-VCL antibody. Blots were quantified and the STAR and CYP19A1/ $\alpha$ VCL ratios are shown. The results are expressed as means  $\pm$  SEM of four independent experiments. Bars with different letters are significantly different ( $P < 0.05$ ).

**Figure 7: Effect of CHEMERIN treatment on the cholesterol content and the HMGCR protein levels and the potential involvement of CMKLR1 in bovine granulosa cells.**

Overnight-starved granulosa cells from small bovine follicles were preincubated with/without 1  $\mu$ g/ml CMKLR1 antibody or IgG for 1 h and then treated for 48h with or without 200 ng/ml hRec  $\pm$   $10^{-8}$  M FSH or  $\pm$   $10^{-8}$  M IGF1 as described in Figure 4. Granulosa cells were lysed and cholesterol content was determined as indicated in the Materials and Methods and expressed as  $\mu$ g/ $\mu$ g total protein. Granulosa cell lysates were also subjected to SDS-PAGE as described in Materials and Methods. The membranes were incubated with antibodies raised against the HMGCR proteins. Equal protein loading was verified by reprobing membranes with an anti-VCL antibody. Blots were quantified and the HMGCR/ $\alpha$ VCL ratios are shown. The results are expressed as means  $\pm$  SEM of four independent experiments. Bars with different letters are significantly different ( $P < 0.05$ ).

**Figure 8: Effect of CHEMERIN treatment on basal and FSH- or IGF1-stimulated phosphorylation of MAPK3/MAPK1 and the potential involvement of CMKLR1 in bovine granulosa cells.**

Granulosa cells from small bovine follicles were cultured for 48 h in medium with serum and then in serum-free medium in the presence or in the absence of hRec (200 ng/ml) for various times (1, 5, 10 and 30 min) (A) or for 48h  $\pm$   $10^{-8}$  M FSH or  $\pm$   $10^{-8}$  M IGF1 with or without one hour of CMKLR1 antibody or IgG preincubation (the same conditions described in Figure 5, (B)). Lysates (50  $\mu$ g) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-MAPK3/MAPK1 and anti-MAPK3 antibodies. Representative blots from three independent experiments are shown. Bands on the blots were quantified and the phosphorylated protein / total protein ratio is shown. The results are reported as means  $\pm$  SEM. Bars with different letters are significantly different ( $P < 0.05$ ).

**Figure 9: Effects of CHEMERIN treatment on bovine oocyte nuclear maturation.**

A. Hoechst (blue) staining before (T0, GV stage just after oocyte collection; 1), during (IVM 10 h, GVBD stage; 2), and after maturation in the absence (IVM 22 h, MII stage; 3) or in the presence of CHEMERIN at a concentration of 200 ng/ml (IVM 22 h, GV stage; 4). Bars = 50  $\mu$ M (1-4). At each stage (panel 1 to 4), the right picture is a magnification (X5) of the left picture

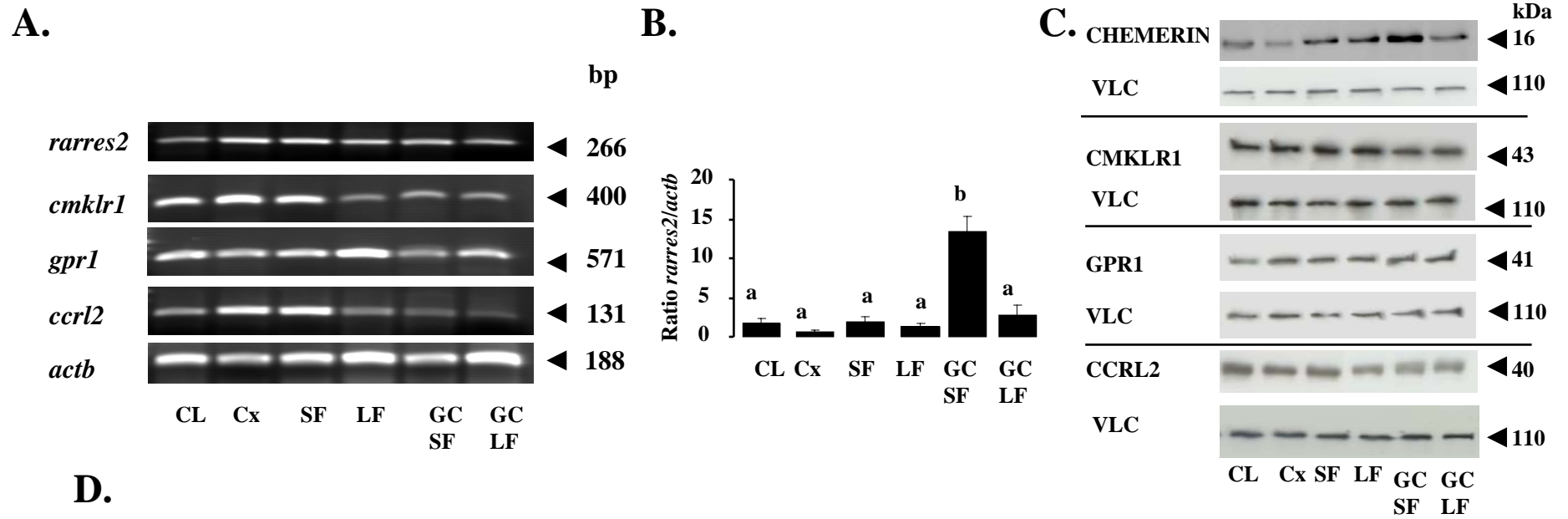
B) Bovine oocytes were allowed to mature for 10 or 22 h in the presence or absence of hRec (200 ng/ml). The percentage of oocytes at the GV stage in the various conditions is shown. Different letters indicate significant differences with  $P < 0.05$ . The results are presented as mean  $\pm$  SEM of three independent experiments. We used at least 50 bovine oocytes for each set of conditions in each experiment. C) Bovine oocytes were allowed to mature for 22 h in the presence or absence of various concentrations of hRec (100, 200 or 400 ng/ml). The percentage of oocytes at the GV stage in the various conditions is shown. Different letters indicate significant differences with  $P < 0.05$ . The results are presented as mean  $\pm$  SEM of three independent experiments. We used 50 bovine oocytes for each set of conditions in each experiment.

**Figure 10: Effects of CHEMERIN treatment on the level of phosphorylation of MAPK3/1 in bovine cumulus cells and oocyte from COCs and on the progesterone levels in culture medium after IVM.**

A. Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of various doses of CHEMERIN (100, 200 and 400 ng/ml). The culture medium was then collected, and its progesterone content was analyzed by RIA, as described in Materials and Methods. Results are expressed as nanograms per milliliter of 50 COC-equivalent cumulus cells. Results are mean  $\pm$  SEM for three independent experiments. Different letters indicate significant differences with  $P < 0.05$ . B and C: Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of CHEMERIN (200 ng/ml). COCs were then mechanically separated into oocyte and cumulus cells. Denuded oocytes (50 oocytes per lane, B) and cumulus cells (C) were lysed and subjected to Western blotting with antibodies against phospho-MAPK3/1 and MAPK3. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein:total protein ratio is shown. Different letters indicate significant differences with  $P < 0.05$ . The results are presented as mean  $\pm$  SEM.

**Table 1: Oligonucleotide Primers Sequences**

Abbrev.	Name	Forward 5'-3'	Reverse 5'-3'	Size bp
<i>ppia</i>	Cyclophilin A	GCATACAGGTCCTGGCATCT	TGTCCACAGTCAGCAATGGT	217
<i>Gpr1</i>	GPR1	CTGTCATTTGGTTCACAGGA	AACAACCTGAGGTCCACATC	571
<i>Rpl19</i>	Ribosomal protein L19	AATCGCCAATGCCAACTC	CCCTTTCGCTTACCTATAACC	156
<i>actb</i>	Beta Actin	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATAACC	180
<i>Cmklr1</i>	CMKLR1	CGGCCATGTGCAAGATCAGC	CAGGCTGAAGTTGTTAAAGC	400
<i>Ccrl2</i>	CCRL2	CGTCATGATCACGTGCAAGA	GCAGGAAGTTGCTGATCTTG	131
<i>Rarres2</i>	RARRES2 or Chemerin	GAGGAGTTCCACAAGCATC	ACCTGAGTCTGTATGGGACA	266



**D.**

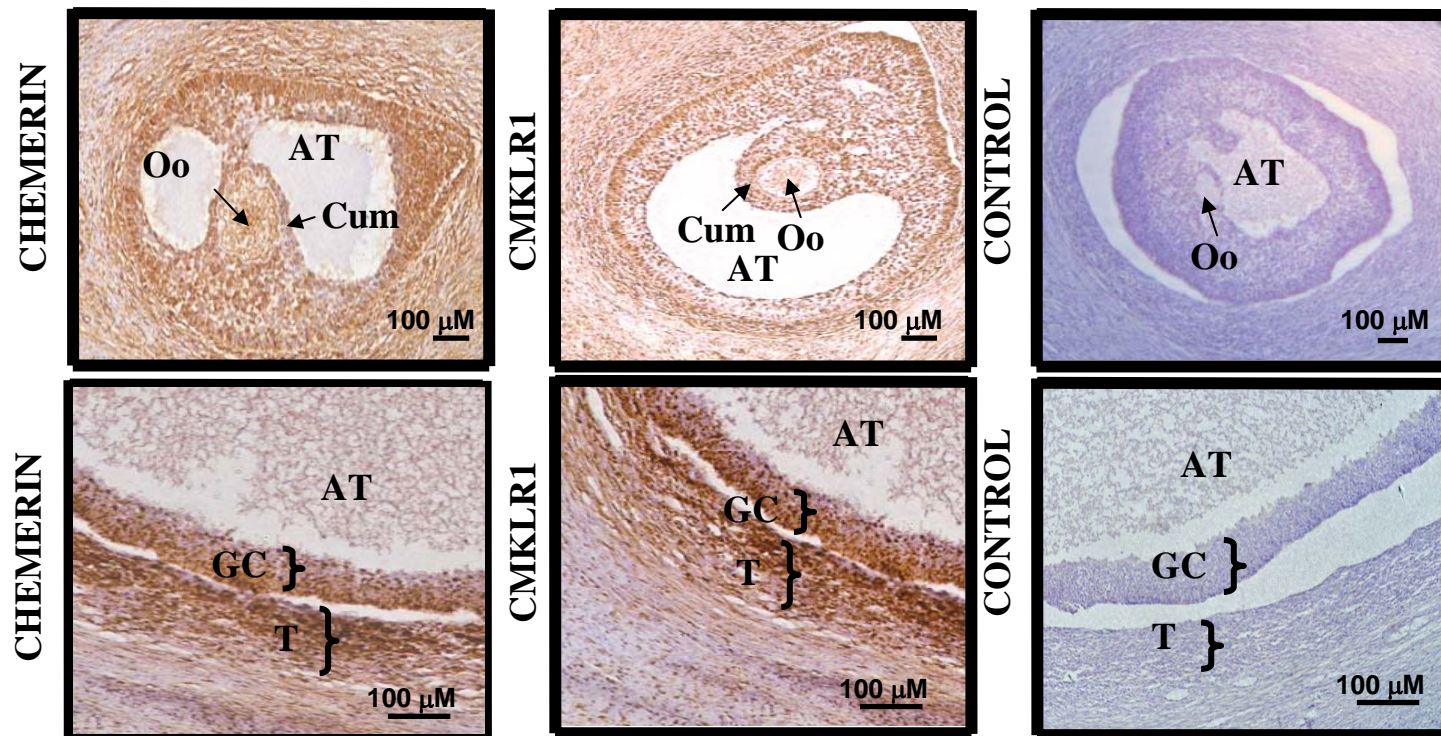


Figure 1

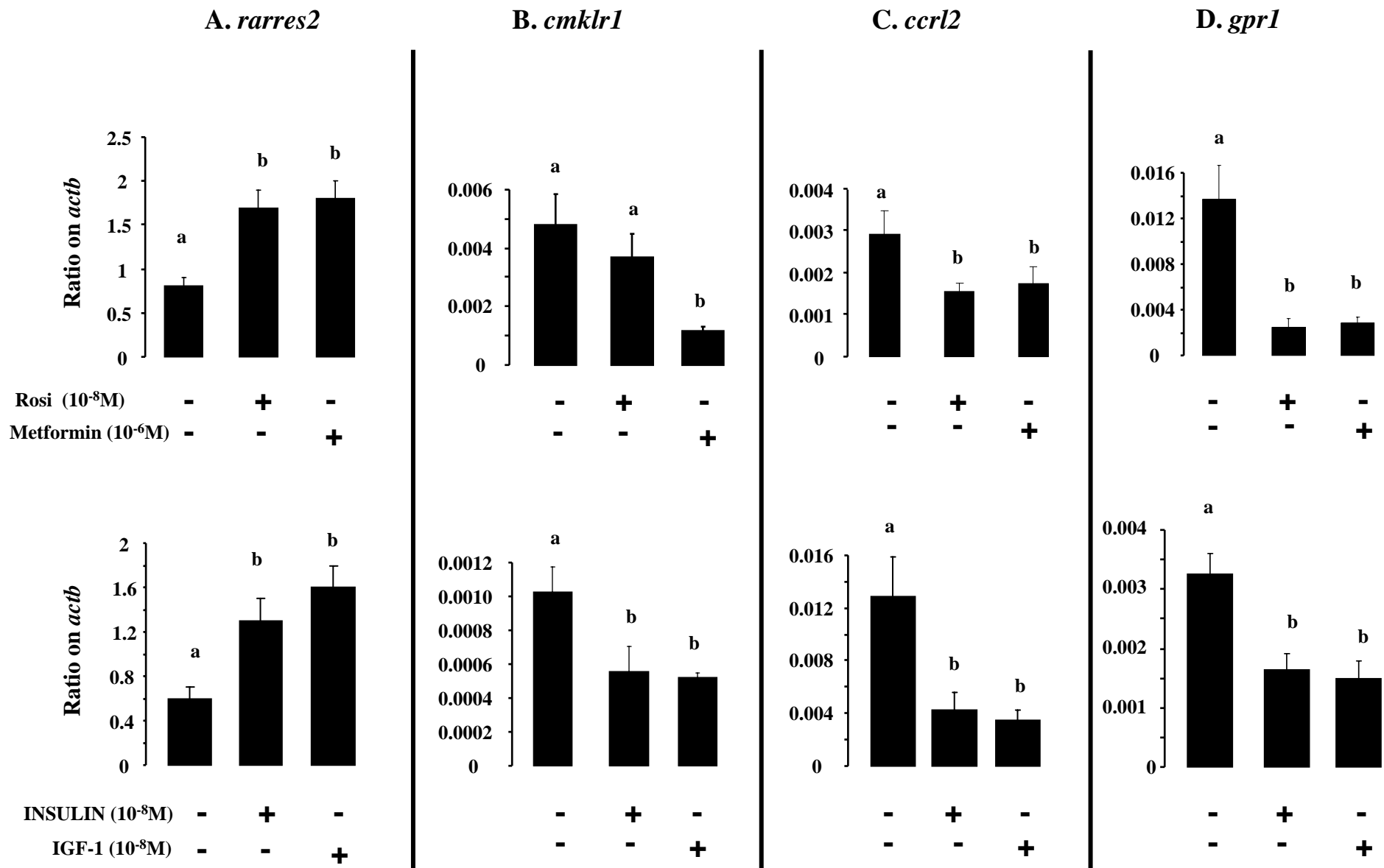
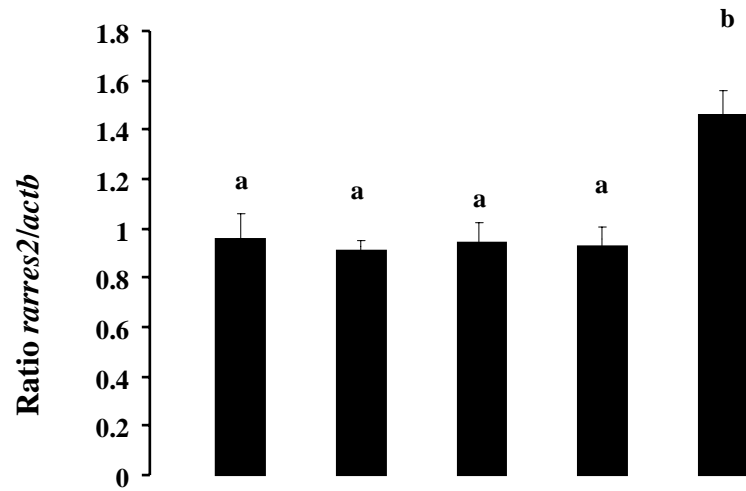


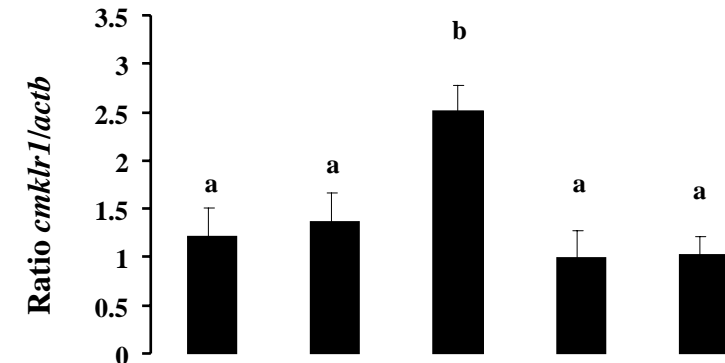
Figure 2

**A. *Rarres2* mRNA**



RESISTIN (100 ng/ml)	-	+	-	-	-
ADIPONECTIN (10 µg/ml)	-	-	+	-	-
LEPTIN (10 ng/ml)	-	-	-	+	-
TNFα (10 ng/ml)	-	-	-	-	+

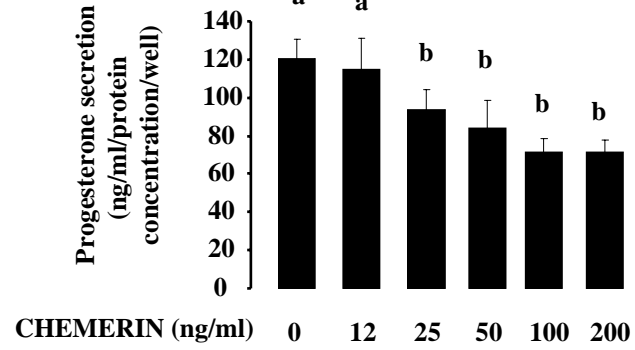
**B. *Cmklr1* mRNA**



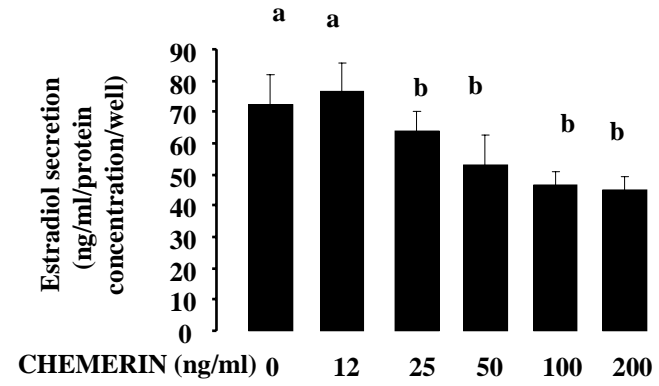
RESISTIN (100 ng/ml)	-	+	-	-	-
ADIPONECTIN (10 µg/ml)	-	-	+	-	-
LEPTIN (10 ng/ml)	-	-	-	+	-
TNFα (10 ng/ml)	-	-	-	-	+

**Figure 3**

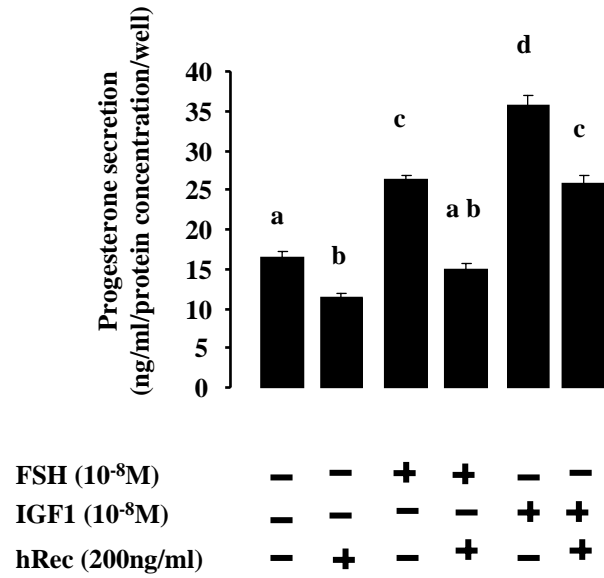
**A.**



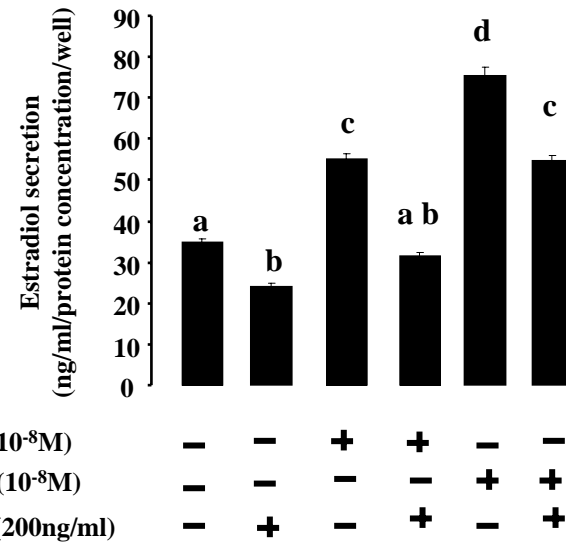
**B.**



**C.**



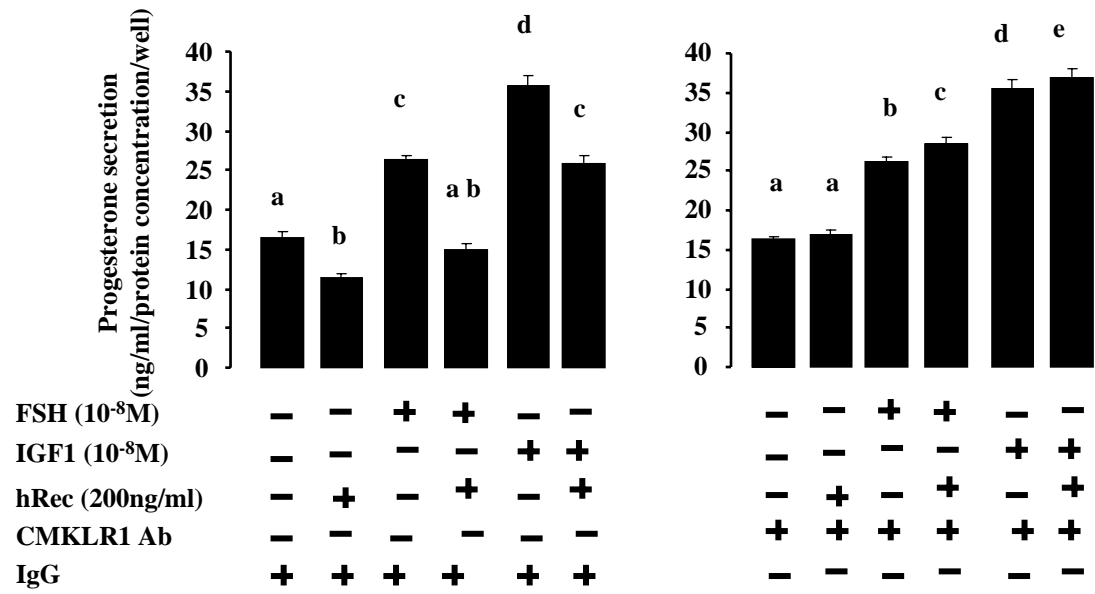
**D.**



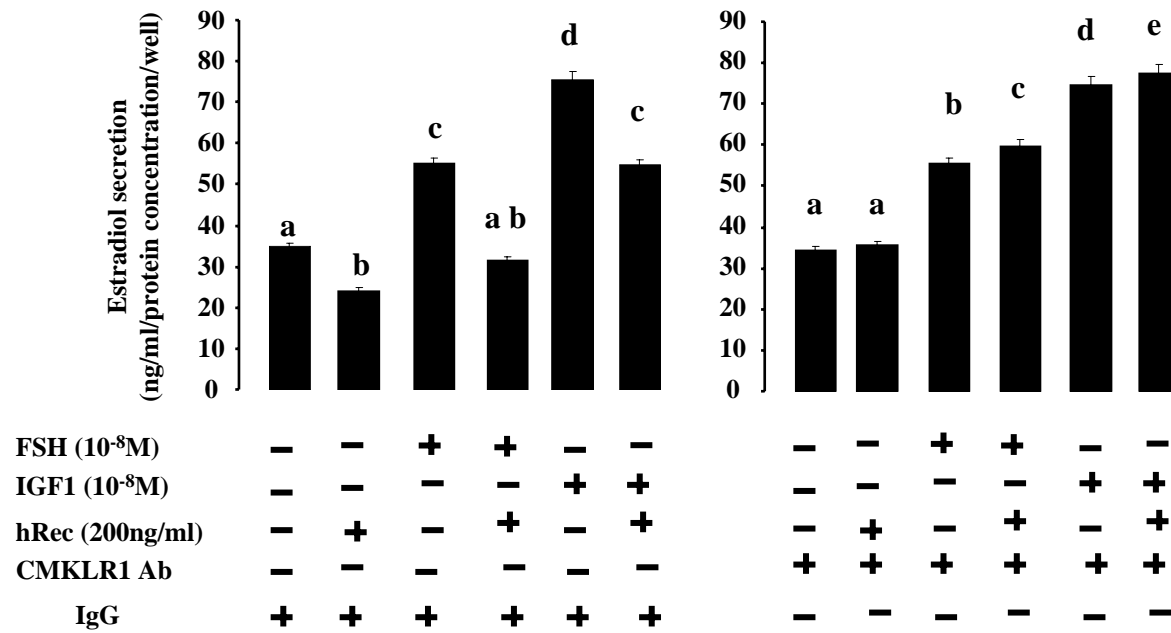
**Figure 4**



**A.**



**B.**



**Figure 5**

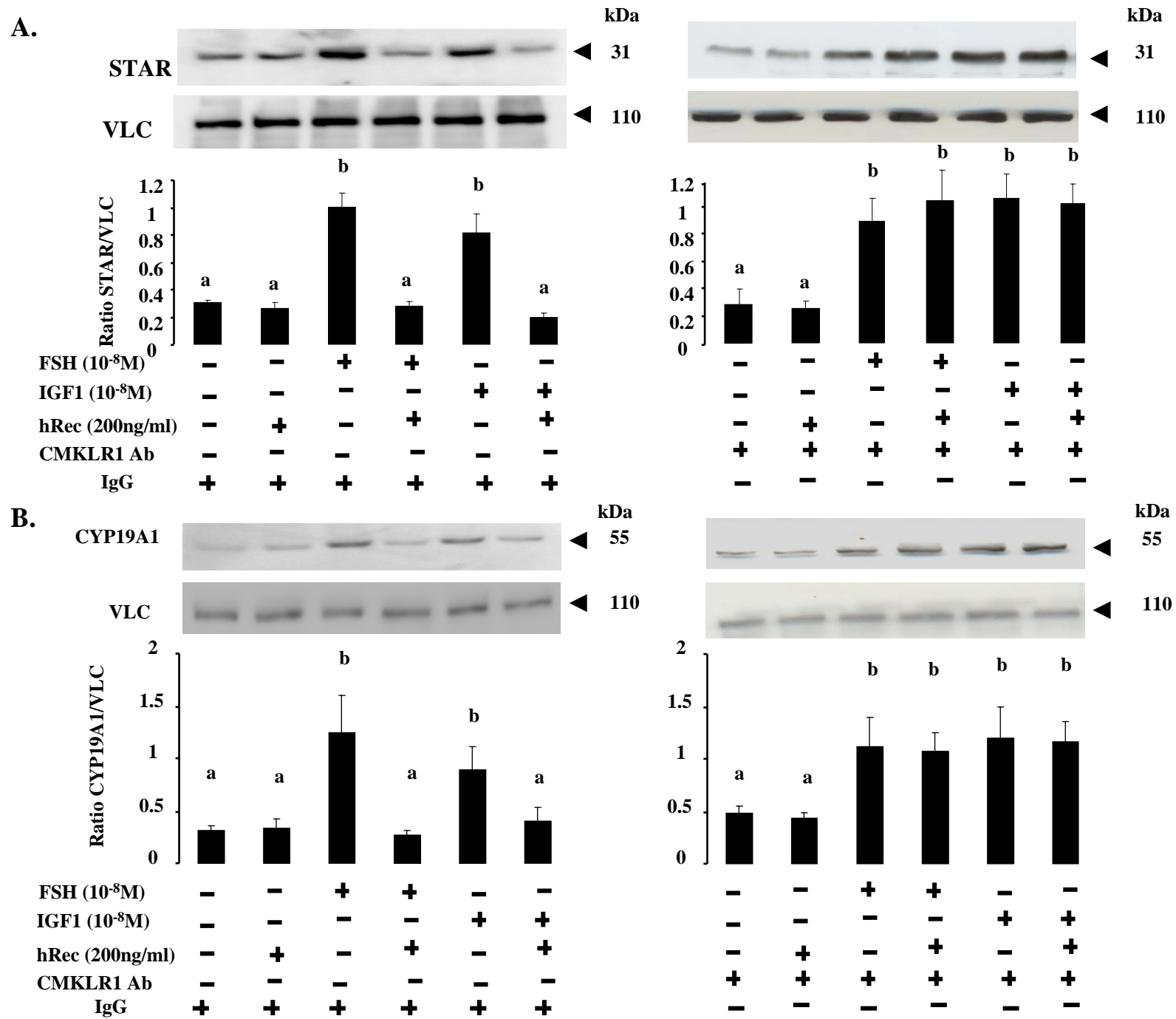
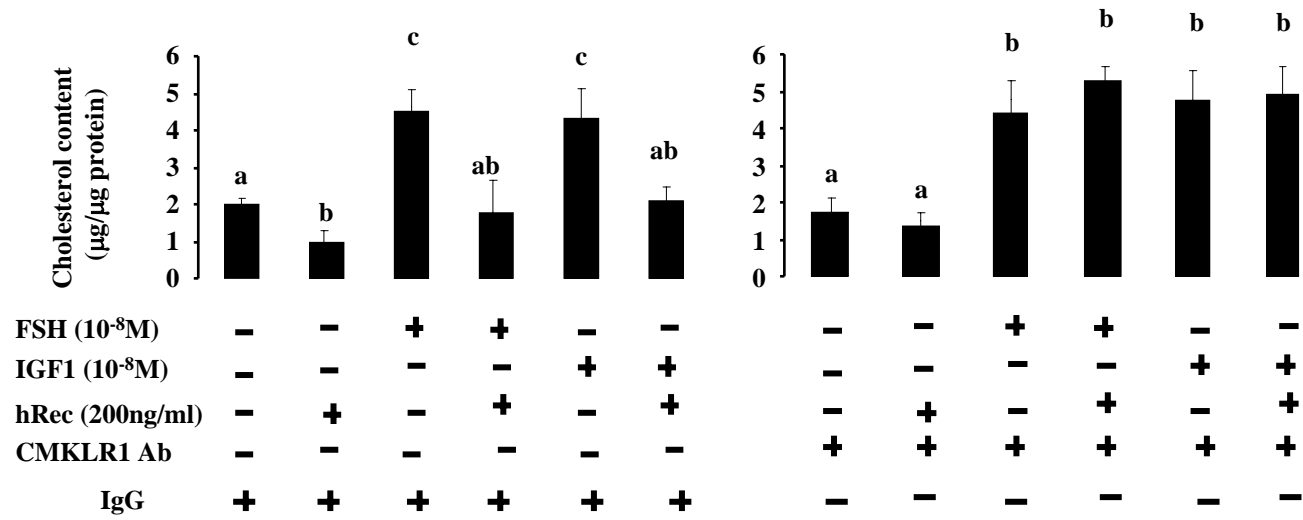
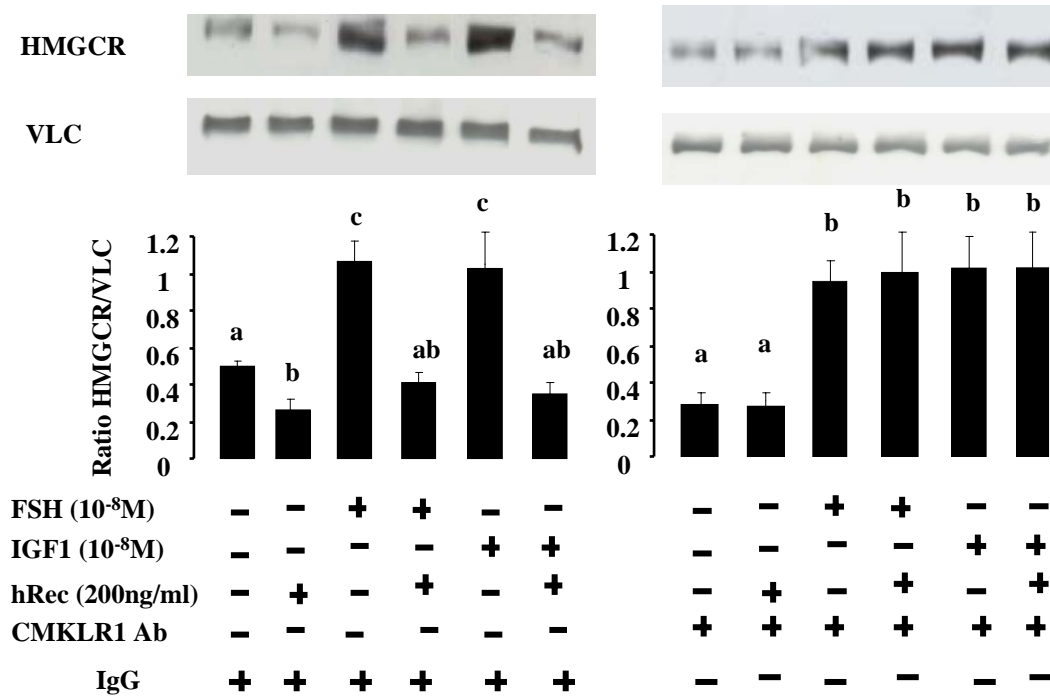


Figure 6

**A.**



**B.**



**Figure 7**

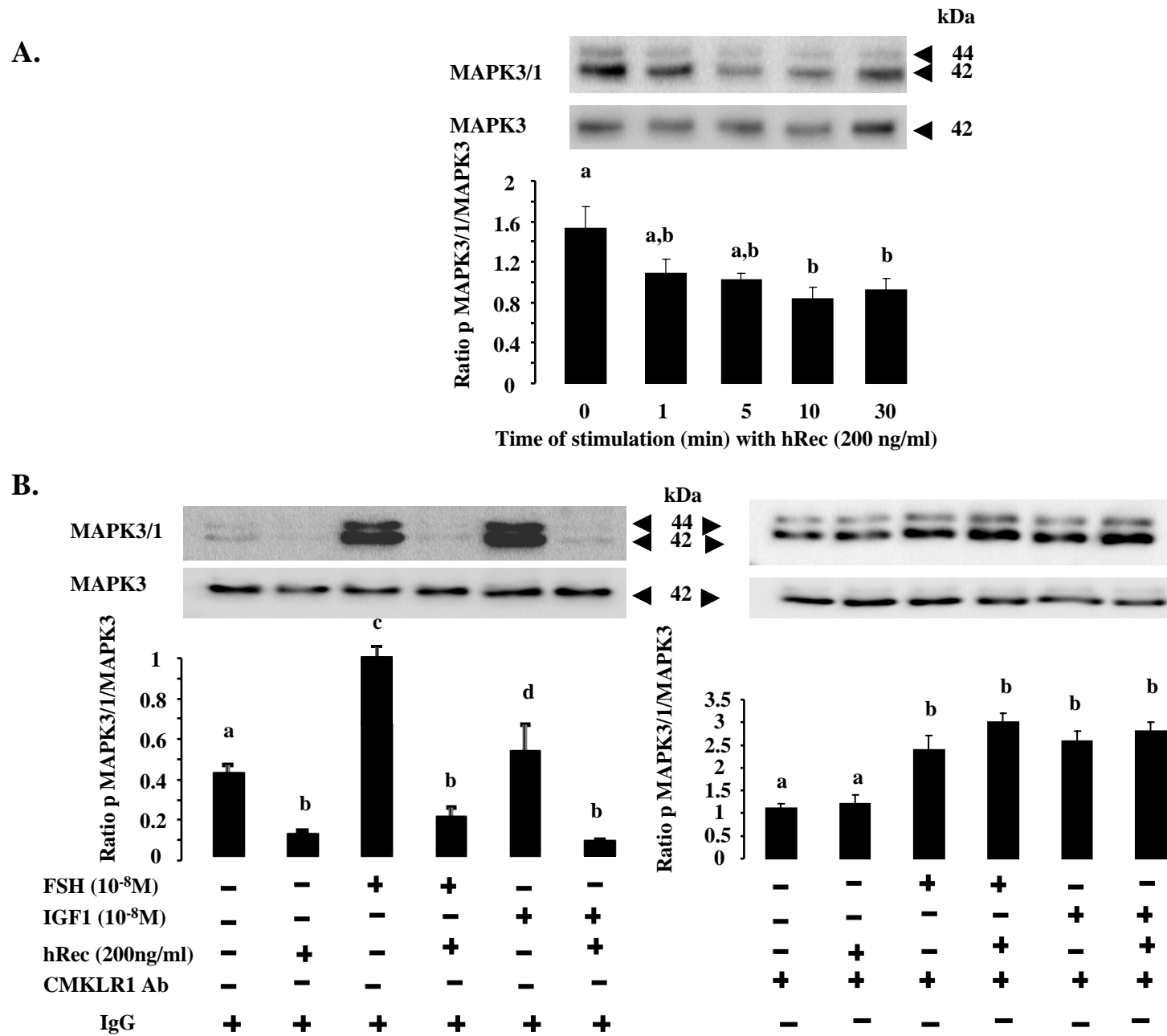
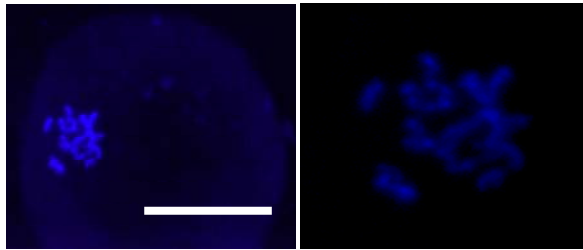


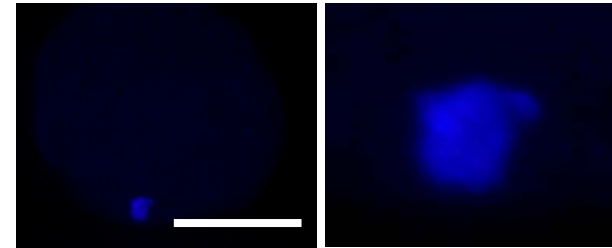
Figure 8

**A.**

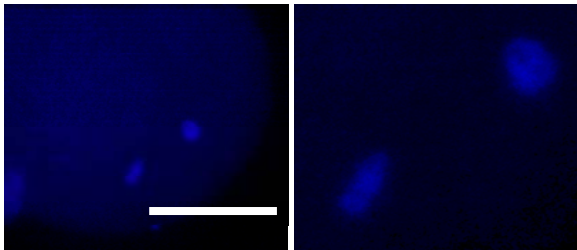
**1. T0 (GV)**



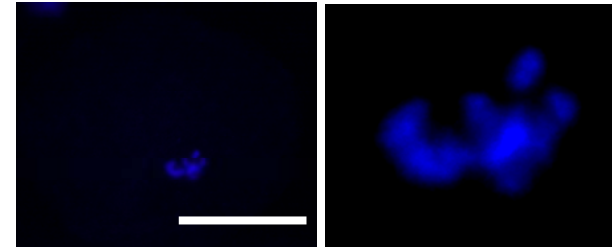
**2. IVM 10h (GVBD)**



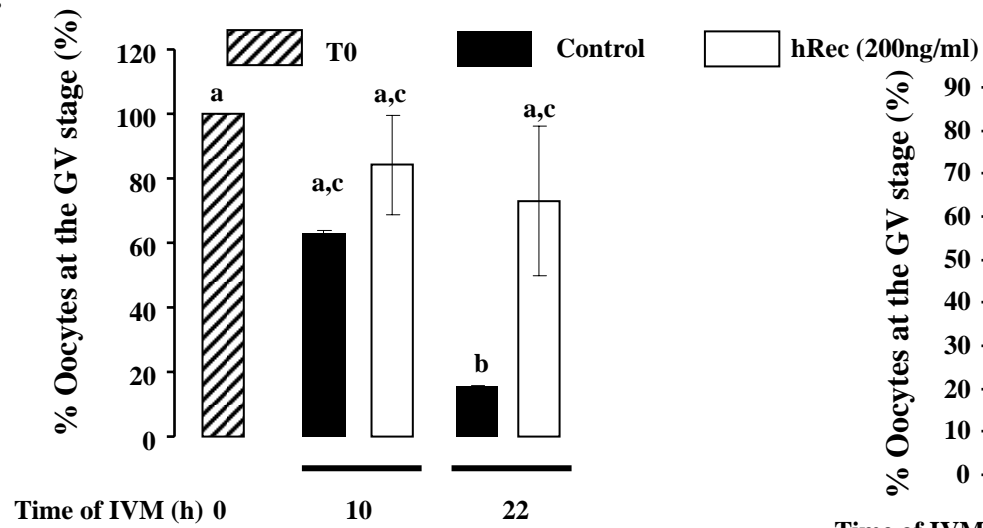
**3. IVM 22h (Meta II)**



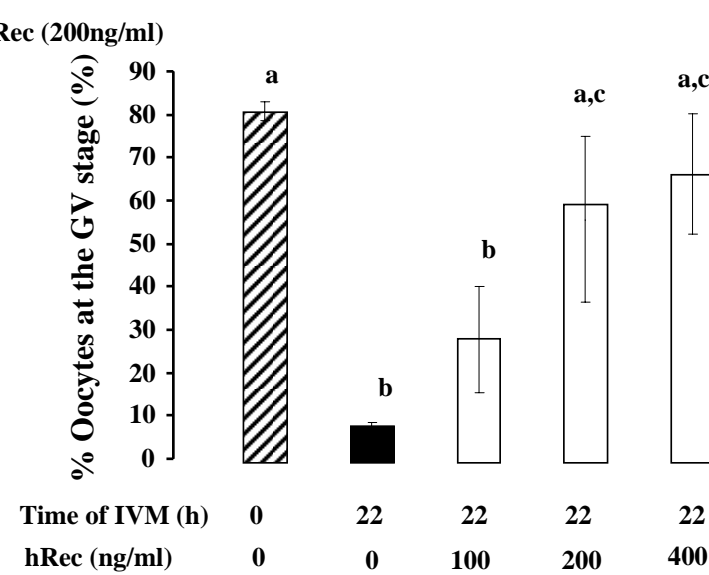
**4. IVM 22h + hRec (200 ng/ml, GV stage)**



**B.**

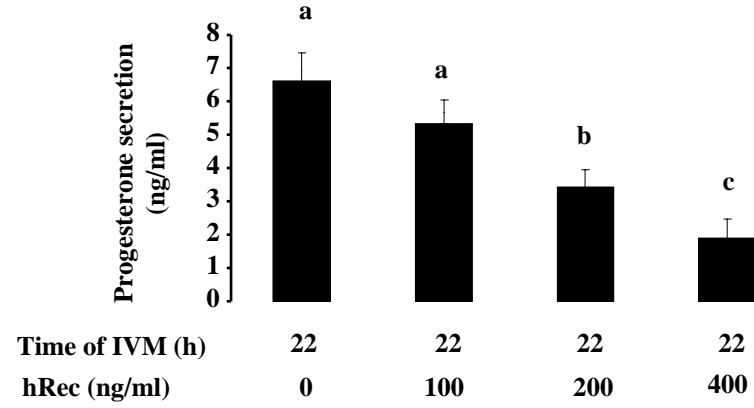


**C.**

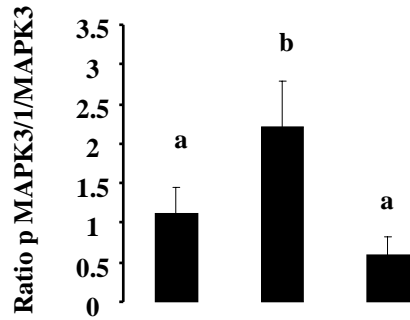
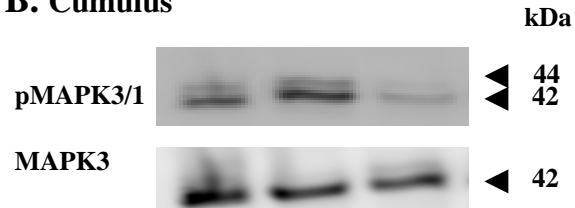


**Figure 9**

**A.**

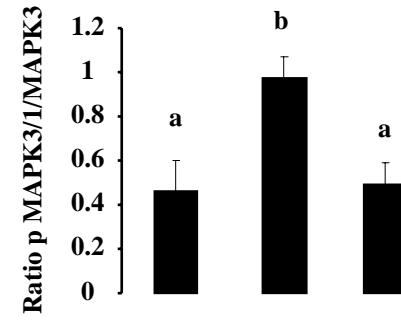
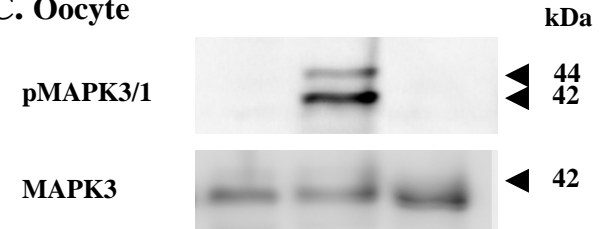


**B. Cumulus**



Time of IVM (h) 0 22 22  
hRec (ng/ml) 0 0 200

**C. Oocyte**



Time of IVM (h) 0 22 22  
hRec (ng/ml) 0 0 200

**Figure 10**