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Apelin (APLN) regulates progesterone secretion and oocyte maturation in bovine ovarian cells

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1 **Apelin (APLN) regulates progesterone secretion and oocyte maturation**
2 **in bovine ovarian cells**

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16
17 **Short Title:** Role of apelin in bovine ovarian follicle

21 **Abstract**

22 APLN and its G-protein coupled receptor APLNR are expressed in the bovine ovary.
23 However, their role in granulosa cells and oocytes is unknown. Here, we studied their
24 expression in bovine ovarian cells and investigated their regulation in cultured luteinizing
25 granulosa cells in response to IGF1 and FSH. We determined the effect and the molecular
26 mechanism of APLN (isoforms 17 and 13) on bovine granulosa cell progesterone secretion
27 and on oocyte maturation. By RT-qPCR and immunoblot, we showed that the expression of
28 both APLN and APLNR in granulosa and oocytes significantly increased with ovarian
29 follicles size whereas it was similar in theca interstitial cells. *In vitro*, in unstimulated
30 luteinizing bovine granulosa cells and in response to IGF1 (10^{-8} M) but not to FSH (10^{-8} M),
31 we observed that APLN (-17 and -13) (10^{-9} M) increased progesterone production; this was
32 abolished in response to the APLNR antagonist ML221. These latter effects were dependent
33 on the MAPK ERK1/2 kinase. Furthermore, we showed that APLN (-17 and -13) (10^{-9} M)
34 increased cell proliferation through AKT signaling. Conversely, the addition of APLN-13 and
35 APLN-17 to *in vitro* maturation medium containing IGF1 (10^{-8} M) but not FSH (10^{-8} M)
36 arrested most oocytes at the germinal vesicle stage, which was associated with a decrease in
37 progesterone secretion, an inhibition in MAPK ERK1/2 phosphorylation and an increase in
38 PRKA phosphorylation in oocytes. Thus, APLN can increase progesterone secretion and cell
39 proliferation in bovine luteinizing granulosa cells *in vitro*, while it blocks meiotic progression
40 at the germinal vesicle stage during bovine oocyte *in vitro* maturation.

41

42 **Keywords:** adipokines, steroidogenesis, growth factors, bovine ovary, signaling

43

44 **Introduction**

45 Apelin (APLN) is a recently described adipokine mainly produced by adipose tissue. It
46 is translated as a 77-amino acid prepropeptide precursor and cleaved into shorter peptides
47 (e.g., APLN-13, APLN-17 and -36) (Hosoya *et al.* 2000). However, APLN-13 displays much
48 greater biological potency than APLN-36 (Tatemoto *et al.* 1998, Kawamata *et al.* 2001).
49 APLN peptides are ligands for the cognate G-protein coupled receptor APLNR that was first
50 defined as being analogous to the angiotensin II type 1 receptor (AT1) (O'Dowd *et al.* 1993).
51 APLN, signaling through APLNR, can modulate numerous intracellular signaling cascades
52 including MAPKs (Bai *et al.* 2008), AKT (Liu *et al.* 2015) and PRKA (Yang *et al.* 2016).
53 APLN/APLNR mRNA and protein have a wide tissue distribution in the central nervous
54 system and in the peripheral organs, including the heart, lung, vessels, and adipose tissue, as
55 well as the testis, ovary (Habata *et al.* 1999) and placenta in human and rodents. The multiple
56 expression sites of the APLN/APLNR system indicate that it may play pivotal roles in various
57 physiological functions. Indeed, APLN is involved in the regulation of the cardiovascular
58 system (Wu *et al.* 2014), energy metabolism (Bertrand *et al.* 2015), food intake (Lv *et al.*
59 2012), angiogenesis (Zhang *et al.* 2016) and neuroendocrine functions (Newson *et al.* 2013).
60 APLN and APLNR have also been identified in the gonads and in the arcuate, supraoptic and
61 paraventricular hypothalamic nuclei, suggesting roles in the control of reproduction (Sandal *et*
62 *al.* 2015).

63

64 Our laboratory has recently described APLN and APLNR in human ovarian cells
65 (Roche *et al.* 2016). In primary human granulosa cells, we have shown that APLN increases
66 IGF1-induced steroidogenesis through an increase in 3-beta-hydroxysteroid dehydrogenase
67 protein expression and activation of the MAPK ERK1/2 and AKT pathways (Roche *et al.*
68 2016). In the rat, a recent study showed that APLN can promote the proliferation of granulosa

69 cells and inhibit apoptosis via PI3/AKT signaling (Shuang *et al.* 2016). In the male rat, an
70 intracerebroventricular injection of APLN-13 reduces testosterone secretion by inhibiting
71 luteinizing hormone (LH) release, suggesting a role for APLN in the control of reproductive
72 functions at both the pituitary and gonad level (Sandal *et al.* 2015). Furthermore, recent data
73 demonstrated that an intraperitoneal injection of APLN-13 in male rats decreases serum
74 testosterone, LH and follicle-stimulating hormone (FSH) levels (Tekin *et al.* 2016). In bovine
75 species, it has been shown that *APLN* and *APLNR* mRNAs are expressed in the bovine follicle
76 and corpus luteum (CL) (Shirasuna *et al.* 2008, Schilffarth *et al.* 2009, Shimizu *et al.* 2009).
77 In the CL, expression decreases at the end of the luteal phase and during CL regression
78 (Shirasuna *et al.* 2008, Schilffarth *et al.* 2009, Shimizu *et al.* 2009). Thus, the APLN/APLNR
79 system could be involved in vascular establishment, maturation and maintenance in the CL
80 during the estrous cycle (Shirasuna *et al.* 2008). In the bovine follicle, APLNR is present in
81 bovine granulosa cells where its expression is increased by progesterone (Shimizu *et al.*
82 2009). In mature follicles, the APLN/APLNR system is thought to play a crucial role during
83 follicle selection and dominance in the bovine species (Shimizu *et al.* 2009). However, the
84 presence and the role of the APLN/APLNR system have never been investigated in bovine
85 granulosa cells and oocytes.

86

87 The main objectives of the present study were to investigate the regulation of APLN
88 and APLNR expression and the effects of APLN on progesterone production and cell
89 proliferation as well as the associated molecular mechanisms in primary bovine granulosa
90 cells. Furthermore, we analyzed the effect of APLN on *in vitro* bovine oocyte maturation.

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95

96 **Materials and Methods**

97 *Ethics*

98 All experimental protocols were approved by an ethics committee (“Comité d’Ethique
99 en Expérimentation Animale Val de Loire”; CEEA VdL, protocol registered as n°2012-09-6),
100 and were carried out in accordance with the guidelines of the French Council for Animal
101 Care.

102 *Hormones and reagents*

103 Recombinant human IGF1 was obtained from Sigma (St Louis, MO, USA). Purified
104 ovine FSH (lot no.AFP-7028D, 4453 IU/mg, FSH activity=175 x activity of (oFSH-S1) used
105 for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda,
106 MD, USA. Recombinant human APLN-13 (rh APLN-13) and 17 (rh APLN-17) were
107 purchased from Abcam (Paris, France) and R&D Systems (Lille, France), respectively.
108 Thymidine methyl- H^3 was obtained from Amersham Life Science (Arlington Heights, IL).
109 The pharmacological inhibitors U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(o-
110 aminophenylmercapto)butadiene monoethanolate) , LY294002 (2-Morpholin-4-yl-8-
111 phenylchromen-4-one), SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-
112 1H-imidazole) and Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-
113 pyrrazolo[1,5-a]-pyrimidine) were obtained from Millipore (Molsheim, France). The APLNR
114 antagonist, ML221 was purchased from R&D Systems.

115

116 *Antibodies*

117 Rabbit polyclonal antibodies to APLN and APLNR were purchased from Antibodies-
118 online and Sigma, respectively. Rabbit polyclonal antibodies to phospho-MAPK ERK1/2
119 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-PRKA (Thr172) and total PRKA
120 were obtained from Ozyme (Montigny Le Bretonneux, France). Mouse monoclonal
121 antibodies to VINCULIN (VCL) were obtained from Sigma (St. Louis, MO, USA). Rabbit
122 polyclonal antibodies recognizing total ERK2 (C14), phospho-AKT (Ser 473) and total
123 MAPK P38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies
124 were used at 1:1,000 for Western blotting.

125

126 ***Collection of bovine follicles and primary culture of bovine granulosa cells***

127 Bovine ovaries were obtained from adult cows collected at a local slaughterhouse and
128 transported aseptically before dissection. Antral follicles were designated into three different
129 groups based on diameter (3–5 mm – small (SF), 6–9 mm–medium (MF) and > 9 mm–large
130 (LF)). In each group of follicles, granulosa (GCs), theca interstitial cells and oocyte for
131 mRNA and protein extraction were collected and immediately frozen in liquid nitrogen and
132 stored at -80°C. For culture, GCs were recovered from small antral follicles (SF) in modified
133 McCoy's 5A medium followed by 5 min of centrifugation. Cells were washed, counted in a
134 hemocytometer and cultured in McCoy's 5A supplemented with 20 mmol/l HEPES, penicillin
135 (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l), 0.1% BSA, 5 mg/l transferrin,
136 20 mg/l selenium, 0.1 µmol/l androstenedione and 10% fetal bovine serum (FBS, PAA
137 laboratories, les Mureaux, France). Approximately 4×10^5 viable cells were seeded in each
138 plastic multiwell containing 1 ml of medium. After 24 h of culture, cells were starved with
139 McCoy's 5A medium containing 1% of FBS overnight and then incubated in fresh culture
140 medium with or without the test reagent for the appropriate period of time. All cultures were
141 performed in a water-saturated atmosphere of 95% air/5% CO₂ at 37°C.

142

143 **RNA isolation and RT-PCR**

144 As described previously (Reverchon *et al.* 2014), total RNA was extracted by using
145 Trizol reagent according to the manufacturer's procedure (Sigma Aldrich, Saint Quentin
146 Fallavier, France). Reverse transcription (RT) and polymerase chain reaction (PCR) were then
147 carried out. RT was performed on RNA amounts corresponding to 10 oocytes or on 1 µg of
148 total RNA from granulosa or theca interstitial cells. Briefly, 1 µg of total RNA was reverse
149 transcribed for 1 h at 37°C in a final reaction volume of 20 µl containing 50 mM Tris-HCl
150 (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 200 µM of each deoxynucleotide triphosphate
151 (Amersham, Piscataway, NJ), 50 pmol of oligo(dT) 15, 5 U of ribonuclease inhibitor and 15
152 U of MMLV reverse transcriptase; 2 µM of each set of specific primers were used. These
153 specific primer pairs were for *APLN* (forward 5'- AAGGCACCATCCGATACCTG -3' and
154 reverse "5'- ATGGGACCCTTGTGGGAGA- 3'), and *APLNR (APJ)* (forward "5'-
155 TCTGGGCCACCTACACCTAT -3' and reverse 5'- ACGCTGGCGTACATGTTG -3'). The
156 PCR conditions were DNA denaturation at 95°C for 5 min, 95°C for 1 min, 58°C for 1 min
157 and 72°C min for 35 cycles before a final extension at 72°C for 10 min. For this, 2 µl of RT
158 reaction mixture was used in a 25 µl final volume containing 10 mM Tris-HCl (pH 9.0), 50
159 mM KCl, 1.5 mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each
160 primer and 1 U of Taq polymerase. The results were viewed on a 1.5% agarose gel stained
161 with ethidium bromide and the amplified DNA was extracted and sequenced by the Genome
162 Express Company (Meylan, France). Consumables for RT-PCR were obtained from Sigma
163 (l'Isle d'Abeau Chesnes, France), and Moloney Murine Leukemia Virus reverse transcriptase
164 and the RNase inhibitor were obtained from Promega (Madison, WI, USA).

165

166 **Real-time polymerase chain reaction (PCR)**

167 After reverse transcription, the bovine cDNAs of ovarian cells were diluted 1:5. Real-
 168 time PCR was performed in a 20 µl final volume containing 10 µl iQ SYBR Green supermix
 169 (Bio-Rad), 0.25 µl of each primer (10 µM), 4.5 µl of water and 5 µl of template. The cDNA
 170 templates were amplified and detected with the MYIQ Cyclor real time PCR system (Bio-
 171 Rad) using the following protocol: 1 cycle for 5 min at 95°C to denature the sample and then
 172 40 cycles, 1 min at 95°C for denaturation, 1 min at 60°C for hybridization, 1 min at 72°C for
 173 stretching and finally 1 cycle for 5 min at 72°C for final elongation. Gene expression was
 174 normalized using the geometric mean of three housekeeping genes (*ACTB*, *RPL19* and *PPIA*)
 175 with stable expression under our conditions (data not shown) The description of the different
 176 primers is: *ACTB* (forward “5’-GCGGGAAATCGTGCGTGACATT-3’ and reverse 5’-
 177 GATGGAGTTGAAGGTAGTTTCGTG-3’) *RPL19* (forward “5’-
 178 GGGCATAGGTAAGCGGAAGG-3’ and reverse 5’-TCAGGTACAGGCTGCCGA -3’) and
 179 *PPIA* (forward “5’- AGCATGTGGTGTGGCAAA -3’ and reverse 5’-
 180 TCGAGTTGTCCACAGTCAGC-3’). For *APLN* and *APLNR*, the same primers described in
 181 the previous paragraph were used. The normalized values of relative expression (R) were
 182 calculated according to the following equation:
$$R = \frac{(E_{Gene}^{-Ct Gene})}{(\text{geometric mean } (E_{Ppia}^{-Ct Ppia}; E_{Rpl19}^{-Ct Rpl19}))}$$

 183 where Ct is the cycle threshold and E is the PCR efficiency for each primer pair. The
 184 specificity of the amplified fragment sequence was assessed by Beckman Coulter Genomics
 185 (Essex, United Kingdom). The efficiency was between 1.7 and 2.

186

187 ***Protein extraction and Western blot***

188 Bovine ovarian cells were homogenized as previously described (Tosca *et al.* 2007a,
 189 Tosca *et al.* 2007b). Lysates were incubated on ice for 30 min before centrifugation at 12,000
 190 x g for 20 min at 4°C. The pellet was eliminated and the samples were stored at -80°C. The
 191 protein concentration for each condition was measured using a BCA protein assay. Samples

192 were denatured and submitted to electrophoresis on 12% SDS-polyacrylamide gels at 90 volts
193 before being transferred onto nitrocellulose membranes (Schleicher and Schuell,
194 Ecquevilland, France). Then, the membranes were blocked for 30 min with TBS-Tween-milk
195 5% and incubated with specific primary antibodies (dilution 1/1000) for 16 h at 4°C. After
196 several washes, membranes were incubated for 1 h 30 min with the secondary antibodies
197 conjugated with HRP anti-rabbit or anti-mouse IgG at a final dilution of 1:5000. Proteins were
198 revealed by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using
199 a G:Box SynGene (Ozyme) with GenSnap software (release 7.09.17). Quantification was
200 performed with GeneTools software (release 4.01.02).

201

202 ***Thymidine incorporation into granulosa cells***

203 Primary bovine granulosa cells were cultured for 24 h in McCoy's 5A medium and
204 10% fetal bovine serum (FBS). Cells were plated in 24-well plates (2×10^5 viable cells/well)
205 and four replicates were tested for each experimental condition (APLN and/or IGF1 in the
206 absence or in the presence of ML221) for each culture. After several washes and overnight
207 serum starvation, cells were cultured for 24 h with $1 \mu\text{Ci}/\mu\text{l}$ of [^3H] thymidine (Amersham
208 Life Science, Arlington Heights, IL) in the presence or absence of APLN 13 or APLN 17
209 and/or IGF1 (10^{-8} M). Thymidine was then removed with PBS and cells were fixed with cold
210 50% trichloroacetic acid for 15 min on ice. Finally, cells were lysed using 0.5 N NaOH and
211 the radioactivity was counted in a β -photomultiplier by adding scintillation fluid (Packard
212 Bioscience).

213

214 ***Progesterone ELISA assay***

215 Progesterone concentrations were measured in serum-free medium from primary
216 bovine granulosa cells after 48 h of culture using an EIA protocol as previously described

217 (Canepa et al. 2008). Cells were plated in 48-well plates (10^5 viable cells/well) and six
218 replicates were tested for each experimental condition (rh APLN-13 or -17, IGF1) in absence
219 or presence of ML221) for each culture. The results were expressed as the concentration of
220 the steroid (ng/ml) according to the protein concentration in each well. The intra- and inter-
221 assay coefficients of variation for progesterone were less than 10% and 11%, respectively.
222 Results are given as mean \pm SEM and are representative of six independent cultures with each
223 condition in quadruplicate.

224

225 ***Bovine oocyte collection and in vitro maturation***

226 Bovine ovaries were collected from a slaughterhouse in sterile NaCl solution and
227 maintained at 37°C until aspiration. The cumulus-oocyte complexes (COCs) were aspirated
228 from follicles 3-8 mm in diameter using an 18-G needle connected to a sterile test tube and to
229 a vacuum line (100 mmHg), as previously described (Reverchon et al. 2014). COCs were then
230 selected under a dissecting microscope. Expanded or non-intact COCs were eliminated: only
231 intact COCs were washed in TCM HEPES 199 (Sigma, Saint Quentin Fallavier, France)
232 supplemented with BSA (0.4%) and gentamicin (2.5 ml/l) under mineral oil (Sigma). The
233 COCs were cultured in TCM 199 (Sigma) with BSA (4 mg/ml) supplemented or not with
234 APLN-13 or APLN-17 (10^{-9} M) in the absence or in the presence of IGF-1 (10^{-8} M) or FSH
235 (10^{-8} M) for 22 h at 39°C in 5% CO₂ in air with saturated humidity. Each oocyte group
236 contained at least 25 oocytes. After maturation, COCs were denuded by pipetting with 0.5%
237 hyaluronidase (Sigma). Collected oocyte and cumulus cells were then frozen at -80°C.

238

239 ***Statistical analysis***

240 All experimental data are presented as the mean \pm SEM. One-way analysis of variance
241 was used to test differences except for the data reported in Figures 3, 4 and 5 for which a two-

242 way analysis of variance was used. $P < 0.05$ was considered to be statistically significant.

243 Statview software was used for all statistical tests.

244

245 **Results**

246

247 ***Expression of APLN and APLNR in different sizes of bovine follicles***

248 Using real time PCR of different sizes of ovarian follicles (SF, MF and LF) we showed
249 that mRNA expression of both *APLN* and *APLNR* in granulosa and oocytes significantly
250 increased with ovarian follicles size whereas it was similar in theca interstitial cells (Figure
251 1.A, B and C). These data were confirmed by immunoblotting (Figure 1.D, E and F). Thus,
252 *APLN* and its receptor, *APLNR* are present in the different bovine ovarian follicular cells
253 (granulosa and theca interstitial cells and oocytes) Furthermore, their expression is differently
254 regulated with the size of follicles.

255

256 ***Effect of IGF1 and FSH on APLN and APLNR expression in bovine granulosa cells***

257 We next investigated the effect of IGF1 (10^{-8} M) and FSH (10^{-8} M), two main
258 hormones involved in the proliferation and differentiation of ovarian cells, on *APLN* and
259 *APLNR* mRNA expression in primary bovine granulosa cells. Overnight-starved cells (with
260 1% FBS) were incubated for 24 h with IGF1 (10^{-8} M) or FSH (10^{-8} M). Using real-time
261 quantitative PCR, we showed that, after 24 h of stimulation, IGF1 increased *APLN* mRNA
262 expression (Figure 2.A), whereas it decreased the mRNA expression of *APLNR* (Figure 2.B).
263 Furthermore, we did not observe any significant effect of FSH on *APLN* or *APLNR* mRNA
264 expression (Figure 2A and B). For *APLN* expression, we confirmed these results at the protein
265 level by immunoblot (Figure 2.C). As shown in figure 2D, we showed that IGF1 but also FSH
266 reduced the *APLNR* protein level. Similar results were observed when cells were incubated
267 for 48h with IGF1 or FSH (Supplemental Figure 1A to D).

268

269 ***Effects of APLN on progesterone secretion by bovine granulosa cells***

270 In order to determine the effect of APLN on the production of progesterone, primary
271 bovine granulosa cells were incubated with various concentrations of APLN-17 or APLN-13
272 (10^{-9} , 10^{-8} and 10^{-6} M) for 48 h (Figure 3.A and C) or with APLN-17 (10^{-9} M) or APLN-13
273 (10^{-9} M) in presence or absence of FSH (10^{-8} M) or IGF1 (10^{-8} M) for 48 h (Figure 3.B and
274 D). Furthermore, to confirm a specific effect of APLN, we pre-incubated or not primary
275 bovine granulosa cells with the APJ antagonist, ML221 in all the conditions described above.
276 As shown in Figure 3.A and C, the secretion of progesterone was increased by either APLN-
277 13 or APLN-17 treatment at a concentration of 10^{-9} M or greater ($P < 0.001$). Two-way
278 ANOVA indicates a significant effect of the concentration of APLN-17 (Figure 3.A) or
279 APLN-13 (Figure 3.C) ($P < 0.05$), a significant effect of the ML221 treatment ($P < 0.05$), and
280 no significant interaction between the concentration used and the ML221 treatment. In the
281 presence of IGF1 (10^{-8} M) but not FSH (10^{-8} M), APLN-13 or APLN-17 (10^{-9} M, 48 h)
282 increased significantly progesterone release ($P < 0.001$) (Figure 3.B and D). As shown in
283 Figure 3.A and B, the APLN-17 induced progesterone secretion in unstimulated cells (Figure
284 3.A) or in response to IGF1 (Figure 3.B) was abolished in the presence of ML221 (10 μ M).
285 Similar results concerning the effect of APLN-13 were obtained (Figure 3.C and D). Two-
286 way ANOVA indicates a significant effect of the treatments (APLN-17, APLN-13 IGF1 and
287 FSH ($P < 0.05$)), a significant effect of the ML221 treatment ($P < 0.05$), and no significant
288 interaction between the various treatments used and the ML221 treatment.

289

290 We next investigated the effect of APLN-13 treatment (10^{-9} M) on the production of
291 progesterone in response to various concentrations of IGF1 (0, 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} M)
292 (Figure 4.A) and FSH (0, 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} M) (Figure 4.C) for 48 h and with IGF1
293 10^{-8} M or FSH 10^{-8} M for various times of stimulation (0 h, 24 h, 48 h, and 72 h) (Figure 4.B
294 and D). As shown in figure 4.A and C, APLN-13 treatment (48h) increased secretion of

295 progesterone whatever the IGF1 concentration ($P < 0.05$) whereas it did not affect the FSH
296 response. Two-way ANOVA indicates a significant effect of the IGF1 (Figure 4.A) and FSH
297 (Figure 4.C) concentration ($P < 0.05$), a significant effect of the APLN-13 treatment only in
298 response to IGF1 but not to FSH ($P < 0.05$), and no significant interaction between the
299 concentration used and the APLN-13 treatment for both FSH and IGF1 experiments (Figure
300 4.A and C). Also, APLN-13 treatment significantly ($P < 0.05$) increased progesterone
301 production in the absence or in the presence of IGF1 for 24 h, 48h, and 72 h whereas it did not
302 affect the FSH response whatever the time of stimulation (Figure 4.B and D). Two-way
303 ANOVA indicates a significant effect of the duration of stimulation with IGF1 (Figure 4.B)
304 and FSH (Figure 4.D), a significant effect of the APLN-13 treatment only in response to IGF1
305 but not to FSH, and no significant interaction between the duration of stimulation and the
306 APLN-13 treatment for both FSH and IGF1 experiments (Figure 4.B and D). Similar results
307 were obtained with APLN-17 treatment (data not shown).

308

309 ***Effects of APLN on granulosa cell proliferation***

310 We also examined the effect of APLN on the number of bovine GCs in culture, either
311 by the induction of mitosis or by altering cell viability. Thymidine methyl- $[^3\text{H}]$ incorporation
312 by primary bovine GCs treated with different concentrations (10^{-8} and 10^{-9}M) of APLN-13
313 (Figure 5.A) or APLN-17 (Figure 5.B) or with APLN-13 or APLN-17 (10^{-9}M) in presence or
314 in absence of IGF1 (10^{-8} M) (Figure 5.C and D) was assessed after 24 h of culture. To
315 confirm a specific effect of APLN, we pre-incubated or not primary bovine granulosa cells
316 with the APJ antagonist, ML221 ($10 \mu\text{M}$) in all the conditions described above. Two-way
317 ANOVA indicates a significant effect of the concentration of APLN-17 (Figure 5.A) or
318 APLN-13 (Figure 5.B) ($P < 0.05$), a significant effect of the ML221 treatment ($P < 0.05$), and
319 no significant interaction between the concentration used and the ML221 treatment. As

320 expected, IGF1 treatment significantly increased thymidine methyl-[³H] incorporation (Figure
321 5.C and D). In the basal state, cell proliferation was increased by APLN-13 and APLN-17
322 treatment (Figure 5.A and B). Furthermore, APLN-13 and APLN-17 also improved thymidine
323 incorporation in response to IGF1 (Figure 5.C and D). All these results were abolished in the
324 presence of ML221 (Figure 5). Two-way ANOVA indicates a significant effect of the
325 treatments (APLN-17 or APLN-13, IGF1, ($P < 0.05$), a significant effect of the ML221
326 treatment ($P < 0.05$), and no significant interaction between the various treatments used and
327 the ML221 treatment. As revealed by staining with trypan blue, neither APLN-13 nor APLN-
328 17 had an effect on cell viability in the absence or presence of IGF1 (data not shown). Thus,
329 in the basal state, APLN-13 or -17 increased both progesterone production and cell
330 proliferation in the absence or in the presence of IGF1. All these data were obtained without
331 affecting cell viability.

332

333 ***Effects of APLN on cell signaling in bovine granulosa cells***

334 It is well-known that APLN can activate various signaling pathways in different cell
335 types ((Bai *et al.* 2008, Liu *et al.* 2015, Yang *et al.* 2016)). Here, we measured the effect of
336 APLN-13 (10^{-9} M) on the phosphorylation levels of mitogen-activated protein kinase (MAPK)
337 ERK1/2 and p38, Akt and PRKA (Figure 6.A to D). As shown in Figure 6. A-C, APLN-13
338 rapidly activated (at 1 or 5 minutes, $P < 0.05$) the MAPK ERK1/2, p38 and Akt signaling
339 pathways. However, this activation was transient. At the opposite, PRKA phosphorylation
340 was significantly decreased after 5 min of APLN-13 stimulation (Figure 6.D). Similar results
341 were observed with APLN-17 (data not shown). Thus, APLN activates MAPK (ERK1/2 and
342 p38) and Akt signaling and inhibits PRKA kinase in primary bovine GCs.

343

344 ***Involvement of MAPK ERK1/2 in APLN-mediated effects on IGF1-induced progesterone***
345 ***secretion***

346 We next investigated the molecular mechanisms involved in the effect of APLN on
347 progesterone production in the absence and in the presence of IGF1 in primary bovine GCs.
348 Cells were incubated in serum-free media supplemented with APLN-13 (10^{-9} M) for 48 hours,
349 which were the same conditions used to measure progesterone production, either in the
350 presence or in the absence of IGF1 (10^{-8} M) treatment for 5 minutes for the kinase
351 phosphorylation studies (Figure 7). As expected, treatment with IGF1 alone increased the
352 phosphorylation of AKT (Figure 7.A) and MAPK ERK1/2 (Figure 7.B), whereas no effect
353 was observed for MAPK P38 (Figure 7.C) and PRKA (Figure 7.D) phosphorylation.
354 Treatment with only APLN- 13 also increased the basal phosphorylation of AKT, MAPK
355 ERK1/2 and MAPK P38 and reduced that of PRKA. Furthermore, an additive effect of
356 APLN-13 and IGF1 was observed for MAPK ERK1/2 and AKT phosphorylation, but no
357 effect was observed for MAPK P38 and PRKA phosphorylation when the two hormones were
358 combined (Figure 7.A to 7.D). To demonstrate the involvement of the MAPK ERK1/2 and
359 AKT signaling pathways in the observed effect of APLN-13 on progesterone secretion in the
360 absence or in the presence of IGF1, we used two pharmacological inhibitors, i.e. U0126 (10
361 μ M) and LY293004 (10 μ M), known to block the MAPK ERK1/2 and AKT signaling
362 pathways, respectively. As shown in Figure 7.E, U0126 but not LY293004 significantly
363 reduced progesterone production in response to APLN-13 in the presence or absence of IGF1.
364 Thus, these data suggest that MAPK ERK1/2 but not AKT is involved in APLN-13-induced
365 progesterone synthesis in response to IGF1.

366

367 ***Involvement of the AKT signaling pathway in APLN-mediated effects on IGF1-induced cell***
368 ***proliferation***

369 We then determined the molecular mechanisms involved in the observed effect of
370 APLN-13 on cell proliferation in response to IGF1 in primary bovine granulosa cells. The
371 cells were pre-incubated in serum-free media with U0126 (10 μ M), LY293004 (10 μ M),
372 SB202190 (10 μ M) and Compound C (10 μ M), known to block the MAPK ERK1/2, AKT,
373 MAPK P38 and PRKA signaling pathways, respectively, and then stimulated the cells or not
374 with IGF1 (10⁻⁸ M) for 24 h. As shown in Figure 8, only LY293004 (10 μ M) significantly
375 reduced APLN-13-induced thymidine incorporation in the basal state and in response to IGF1.
376 Thus, these data suggest that the AKT signaling pathway is involved in APLN-13-induced
377 cell proliferation in response to IGF1.

378

379 ***Effects of APLN on in vitro bovine oocyte maturation***

380 We also studied the effects of APLN-13 and APLN-17 on the meiotic progression of
381 bovine oocytes in cumulus oocyte complexes (COCs) during *in vitro* maturation (IVM) in the
382 absence or in the presence of IGF1 (10⁻⁸M) or FSH (10⁻⁸M). For the control group, after 22 h
383 of culture in IVM medium, most oocytes underwent germinal vesicle breakdown (GVBD) in
384 the absence or in the presence of IGF1 (10⁻⁸M) or FSH (10⁻⁸M) about 95% of oocytes had
385 progressed to the metaphase II stage, with less than 10% remaining at the GV stage (Figure 9.
386 A). Conversely, if COCs matured in the presence of APLN-13 (10⁻⁹M), meiotic progression
387 was inhibited except if FSH (10⁻⁸M) was present in the IVM medium (Figure 9.A). Indeed, in
388 COCs matured for 22 h in IVM medium supplemented with APLN-13 (10⁻⁹M) in the absence
389 or in the presence of IGF1 (10⁻⁸M), about 65% of oocytes remained at the GV stage (Figure
390 9.A). Similar results were observed with APLN-17 (10⁻⁹ M, Figure 9.C). Thus, APLN
391 treatment of COCs during IVM resulted in meiotic arrest in the absence or in the presence of
392 IGF1. Progesterone secretion by cumulus cells is known to play a key role in bovine oocyte
393 maturation (Zhang & Armstrong 1989, Borman *et al.* 2004). We therefore investigated the

394 effects of APLN treatment (APLN-13 (Figure 9.B) and APLN-17 (Figure 9.D)) on
395 progesterone secretion by COCs. The addition of APLN-13 or APLN-17 to the maturation
396 medium for 22 h significantly decreased progesterone secretion in COCs in the absence or in
397 the presence of IGF1 but not in the presence of FSH (Figure 9.B).

398 We investigated the molecular mechanisms involved in the effects of APLN on the
399 nuclear maturation of bovine oocytes in COCs by determining the levels of MAPK ERK1/2
400 and PRKA phosphorylation in the presence or absence of APLN-13 (10^{-9} M) in COCs allowed
401 to mature *in vitro* for 22 h. MAPK ERK1/2 and PRKA are two crucial signaling pathways
402 involved in the bovine oocyte maturation (Tosca *et al.* 2007). As expected and as shown in
403 Figure 10.A and B, the level of MAPK ERK1/2 phosphorylation increased and those of
404 PRKA decreased in the oocytes from COCs during IVM in the absence or in the presence of
405 IGF1 or FSH (Tosca *et al.* 2007). The addition of APLN-13 (10^{-9} M) in the absence or in the
406 presence of IGF1 to the maturation medium for 22 h significantly decreased MAPK ERK1/2
407 phosphorylation, whereas it increased PRKA phosphorylation in oocytes from COCs.
408 However, the APLN-13 treatment did not affect the FSH effect on MAPK ERK1/2 and PRKA
409 phosphorylation. Similar results were observed in response to APLN-17 treatment (data not
410 shown). Thus, APLN treatment during IVM decreased MAPK ERK1/2 and increased PRKA
411 phosphorylation in the absence or in the presence of IGF1 in oocytes from COCs.

412

413

414 Discussion

415 In the present study, we identified APLN and APLNR in bovine ovarian cells from
416 different sizes of follicle. In cultured granulosa cells, we observed that their expression was
417 differently regulated by IGF1, an important hormone involved in the differentiation of ovarian
418 follicles. We also showed that APLN (isoforms-13 and -17) increased progesterone
419 production and cell proliferation through the MAPK ERK1/2 and AKT signaling pathways,
420 respectively. Conversely, we demonstrated that APLN-13 and APLN-17 in basal state or in
421 response to IGF1 decrease *in vitro* oocyte maturation, which was associated with a decrease in
422 progesterone production by COCs in IVM medium, a reduction in MAPK ERK1/2
423 phosphorylation and an increase in PRKA phosphorylation in oocytes.

424 APLN-APLNR has been described in many organs including the brain, uterus and
425 ovary (O'Carroll *et al.* 2013), suggesting a role in reproductive functions. In the bovine ovary,
426 they are found in follicles during final growth up to the preovulatory stage (Schilffarth *et al.*
427 2009). In the present study, we showed that APLN and APLNR are expressed not only in
428 granulosa and theca interstitial cells, as already observed (Schilffarth *et al.* 2009, Shimizu *et*
429 *al.* 2009), but also in oocytes. Furthermore, we demonstrated that APLN and APLNR
430 expression in granulosa and oocytes significantly increased with ovarian follicles size whereas
431 it was unchanged in theca interstitial cells. Other adipokines such as NAMPT (Reverchon *et*
432 *al.* 2016), RARRES2 (Reverchon *et al.* 2014) and ADIPOQ (Maillard *et al.* 2010) have been
433 reported in bovine cumulus oocyte complexess. However, the role of APLN-APLNR in the
434 oocyte remains to be determined. In cultured granulosa from small follicles, we demonstrated
435 that IGF1 but not FSH increases APLN expression, whereas it decreases the expression of
436 APLNR. IGF1 is an intraovarian growth factor that plays a key role in the control of follicular
437 development in mammals (Silva *et al.* 2009). More precisely, IGF1 is known to stimulate
438 granulosa cell proliferation and progesterone production (Reverchon *et al.* 2016). In bovine

439 granulosa cells, an increase in the expression of *APLNR* receptor mRNA is associated with
440 follicular atresia (Shimizu *et al.* 2009). Thus, the negative effect of IGF1 on *APLNR* mRNA
441 expression could explain the positive effect of IGF1 on cell survival. Another study has also
442 shown that progesterone stimulates the expression of *APLNR* in granulosa cells (Shimizu *et*
443 *al.* 2009). Thus, *APLNR* can be regulated by different metabolic and reproductive hormones.
444 In PCOS patients, in our laboratory, we observed that follicular fluid APLN-13 levels and
445 granulosa *APLN* and *APLNR* mRNA expression were higher than those observed in control
446 patients (Roche *et al.* 2016). In humans, the link between serum APLN and PCOS is unclear.
447 Indeed, some studies have described higher serum APLN levels in PCOS patients (Cekmez *et*
448 *al.* 2011, Goren *et al.* 2012, Sun *et al.* 2015), whereas others have reported an inverse
449 association (Altinkaya *et al.* 2014). Moreover, according to these studies, a significant
450 correlation between serum APLN and glucose and insulin is not always observed, suggesting
451 that APLN cannot be used as a marker of insulin sensitivity. These contradictory data can be
452 explained by some differences in ethnicity, age, study design and sample size.

453 In the present study, we showed that the APLN-13 and APLN-17 isoforms increased
454 basal and IGF1-induced progesterone secretion by cultured bovine granulosa cells. This effect
455 was abolished when cells were pre-incubated with the *APLNR* antagonist ML221, suggesting
456 a specific effect of APLN. Furthermore, using different pharmacological inhibitors, we
457 observed that these effects were mediated through the MAPK ERK1/2 signaling pathways. In
458 male rats, it has been reported that that an intracereboventricular infusion of high dose APLN-
459 13 decreases testosterone release by suppressing luteinizing hormone secretion (Sandal *et al.*
460 2015). In that study, the number of Leydig cells decreased; however, no direct effect of APLN
461 on steroidogenesis has been described in this species. In bovine species, no studies have yet
462 investigated the effect of APLN on gonadotropin secretion. However, all these data suggest
463 that APLN may affect reproductive functions at different levels of the hypothalamo-pituitary-

464 gonadal axis. In bovine species, several adipokines have already been described to regulate
465 steroid secretion by granulosa cells in the basal state or in response to IGF1 or insulin. For
466 example, ADIPOQ (Maillard *et al.* 2010) but also RARRES2 (Reverchon *et al.* 2014)
467 decrease insulin/IGF1-induced granulosa cell steroidogenesis, whereas NAMPT increases it
468 (Reverchon *et al.* 2016). In the present study, we showed that the positive effect of APLN on
469 progesterone secretion on bovine granulosa cells was mediated by the activation of MAPK
470 ERK1/2. This latter signaling pathway is well-known to be involved in the regulation of the
471 ovarian steroidogenesis in different species, including the rat ((Tosca *et al.* 2005) and cow
472 (Tosca *et al.* 2007a).

473 We demonstrated for the first time that APLN and its receptor are expressed in bovine
474 ovarian cells. However, it remains to show that APLN produced by these cells is released and
475 active. Indeed, while we showed in primary granulosa cells that the expression levels of
476 APLN were increased by addition of IGF1, the APLNR antagonist ML221 did not show any
477 significant effects on progesterone production or cell proliferation unless cells were
478 stimulated with recombinant human APLN. We tried to measure the APLN concentration in
479 the conditioned culture medium by ELISA assay. However, the levels were undetectable
480 suggesting that although ovarian cells may express APLNR and may be capable of responding
481 to exogenous APLN protein, endogenous levels of APLN protein production may not be
482 sufficient to regulate the cell functions. Thus, one hypothesis is that the APLN effects on the
483 ovarian cells would be more systemic than local. However, it remains to be demonstrated by
484 developing mice lacking APLN specifically in oocyte or granulosa cells.

485 We observed that APLN (the 13 and 17 isoforms) increased cell proliferation in the
486 basal state and in response to IGF1 through the PI3K/AKT signaling pathway, whereas APLN
487 did not affect apoptosis in bovine granulosa cells. Our results are in good agreement with
488 recently described findings in the rat (Shuang *et al.* 2016) showing that APLN promotes

489 granulosa cell proliferation. Other studies have also shown a positive effect of APLN-13 on
490 cell growth in the breast cancer cell line MCF-7 (Peng *et al.* 2015) and the lung
491 adenocarcinoma cell line A549 (Yang *et al.* 2014). In the present study, we did not observe
492 any effect of APLN (13 or 17) on apoptosis in granulosa cells. These data contrast those
493 previously described in bovine granulosa cells (Shimizu *et al.* 2009). However, in that study,
494 the authors did not incubate bovine granulosa cells with APLN, but rather associated the two
495 events (apoptosis and variations in APJ expression). APLN is well-known to exert insulin
496 mimic effects and consequently regulate energy metabolism and insulin sensitivity (Dray *et*
497 *al.* 2008, Yue *et al.* 2010). Since IGF1 and IGF1R have a similar structure to those of insulin
498 and the insulin receptor (Dupont *et al.* 2003), APLN may increase IGF1 sensitivity in
499 granulosa cells as already described in muscle in mice (Dray *et al.* 2008) and consequently
500 improve not only steroidogenesis but also proliferation.

501 For the first time, we have shown the presence of APLN and APLNR in the bovine
502 oocyte. Furthermore, we observed that APLN-13 and APLN-17 decreased *in vitro* oocyte
503 maturation and this was associated with a reduction in progesterone levels in IVM medium
504 and variations in MAPK ERK1/2 and PRKA phosphorylation levels in oocytes. APLN may
505 block bovine nuclear oocyte maturation through the inhibition of progesterone secretion by
506 COCs. Indeed, various studies have reported that progesterone is crucial for oocyte
507 maturation (Zhang & Armstrong 1989, Borman *et al.* 2004). Furthermore, the strong
508 reduction in APLN-induced MAPK ERK1/2 phosphorylation observed in bovine oocytes
509 could also contribute to explaining the inhibitory effect of APLN on oocyte maturation.
510 Indeed, an increase in MAPK ERK1/2 phosphorylation in oocytes at the beginning of
511 maturation has been described in different species, including bovine species (Fissore *et al.*
512 1996). Furthermore, in parallel with the inhibition in APLN-induced MAPK ERK1/2, we
513 observed an increase in PRKA phosphorylation in oocytes that could contribute to explain the

514 blockage of APLN in oocyte maturation. Several data showed that pharmacological activation
515 of PRKA blocks nuclear oocyte maturation in cattle (Bilodeau-Goeseels *et al.* 2007, Tosca *et*
516 *al.* 2007b). In our study, we observed that APLN-13 increased basal and IGF1-induced
517 progesterone production in cultured granulosa cells, whereas it reduced progesterone
518 production by COCs in *in vitro* maturation medium. In COCs, progesterone is produced by
519 cumulus cells, which are also granulosa cells. However, some studies have shown that mural
520 granulosa cells and cumulus cells have differences in their gonadotrophin receptor content
521 and in progestin secretion responsiveness to gonadotrophins (Magnusson *et al.* 1982).
522 Furthermore, it will be interesting to compare the expression and the molecular mechanism of
523 APLN/APLNR in mural granulosa and cumulus cells. The culture conditions (medium,
524 attached cells for mural granulosa cells and cell suspension for COCs) could also explain the
525 contradictory data regarding the effect of APLN on mural granulosa cells and cumulus cells.
526 Indeed, the contrasting data on progesterone production and MAPK ERK1/2 phosphorylation
527 between mural granulosa cells and cumulus cells could be related to the 10% bovine fetal
528 serum in the culture medium used for the seeding of mural granulosa cells in order to improve
529 the cell attachment. Indeed, it is well known that the use of serum induces luteinization of
530 cells thus not representing the physiology of a healthy growing follicle (Gutierrez *et al.* 1997).

531 In conclusion, APLN and its receptor are present in bovine ovarian cells including
532 granulosa cells and oocytes. We showed that APLN can increase progesterone secretion from
533 luteinizing granulosa cells, whereas it inhibits oocyte maturation and progesterone secretion
534 from cumulus cells *in vitro*. In the face of the lack of influence of APLN blockers alone on the
535 parameters assessed herein and limitations of the *in vitro* culture system, further studies are
536 necessary to confirm a physiological role for APLN in the control of folliculogenesis.

537

538

539 **Declaration of interest:**

540 No conflict of interest

541

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554

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- 691
- 692

1 **Figure legends**

2 **Figure 1: APLN and APLNR expression in granulosa, theca interstitial cells and**
 3 **oocytes from small (SF), medium (MF) and large (LF) ovarian bovine follicles. A to C)**

4 *APLN* and *APLNR* mRNA expression was measured by quantitative RT-PCR in different
 5 ovarian cells from SF, MF and LF as described in materials and methods. **D to F) APLN and**
 6 **APLNR protein levels by immunoblotting in granulosa, theca interstitial cells and**
 7 **oocytes from SF, LF and CL.** APLN and APLNR protein levels were quantified in different
 8 ovarian cells from SF, MF and LF as described in materials and methods. Vinculin (VCL)
 9 was used as a loading control. A to F : For granulosa and theca cells for each group of follicle,
 10 a pool of 4 follicles per animal was used and 6 different animals were studied. For oocytes of
 11 each group of follicle, 6 pools each containing 10 (for mRNA, A to C) or 50 oocytes (for
 12 immunoblot, D to F) from various animals were studied. Statistical analyses were separately
 13 performed for each protein (APLN and APLNR). The results are expressed as the mean \pm
 14 SEM. Bars with different letters are significantly different ($P < 0.05$).

15
 16
 17 **Figure 2 : Effect of IGF1 and FSH on *APLN* and *APLNR* mRNA (A, B) and protein (C,**
 18 **D) expression levels in primary bovine granulosa cells. (A, B) *APLN* and *APLNR* mRNA**

19 expression was measured by quantitative RT-PCR in primary bovine GCs after 24 hours of
 20 stimulation with or without IGF1 (10^{-8} M) or FSH (10^{-8} M). The results represent four
 21 cultures of GCs and are presented as mean \pm SEM. Different letters indicate significant
 22 differences at $P < 0.05$. **(C,D)** Granulosa cell lysates were also subjected to immunoblotting
 23 using antibodies raised against the APLN and APLNR protein. Equal protein loading was
 24 verified by reprobing membranes with an anti-VCL antibody. Blots were quantified and the

25 APLN and APLNR to VCL ratio are shown. The results are expressed as the mean \pm SEM of
26 four independent cultures. Bars with different letters are significantly different ($P < 0.05$).

27

28 **Figure 3 : Effect of APLN-17 (A, B) and APLN-13 (C, D) on basal and FSH- or IGF1-**

29 **stimulated secretion of progesterone by bovine granulosa cells.** Granulosa cells from small

30 bovine follicles were cultured for 24h in medium with serum and then in serum-free medium

31 in presence or absence of various doses of APLN-17 (A) or APLN-13 (C) for 48h or in

32 presence or absence of 10^{-9} M APLN-17 or APLN-13 $\pm 10^{-8}$ M FSH or $\pm 10^{-8}$ M IGF1 (B and

33 D) with or without the APJ antagonist, ML221 (10 μ M) as described in *Materials and*

34 *Methods*. The culture medium was then collected and analyzed for progesterone content by

35 Elisa assay. Results are expressed as ng/ml per well. Results are means \pm SEM of three

36 independent experiments. Bars with different letters are significantly different ($P < 0.05$). A

37 and C. Different capital letters indicate a significant effect of the APLN-17 (A) or APLN-13

38 (B) concentration whereas lower case letters indicate a significant effect of the ML221

39 treatment. B and D. Different capital letters indicate a significant effect of treatments (APLN-

40 13 or -17, IGF1 or FSH) whereas lower case letters indicate a significant effect of the ML221

41 treatment.

42

43 **Figure 4 : Effect of APLN-13 treatment on progesterone secretion by bovine granulosa**

44 **cells in response to different doses and times of stimulation of IGF1 and FSH .**

45 Granulosa cells from small bovine follicles were cultured for 24h in medium with serum and

46 then in serum-free medium in presence or absence of various doses of IGF1 (A) or FSH (C),

47 for 48h or for various times with IGF1 (10^{-8} M) (B) or FSH (10^{-8} M) (D) with or without

48 APLN-13 treatment (10^{-9} M) as described in *Materials and Methods*. The culture medium was

49 then collected and analyzed for progesterone content. The results are expressed as protein

50 concentration per well (ng/ml). The results are presented as the mean \pm SEM of three
 51 independent cultures. Bars with different letters are significantly different ($P < 0.05$). A and C.
 52 Different capital letters indicate a significant effect of the IGF1 (A) or FSH (C) dose whereas
 53 lower case letters indicate a significant effect of the APLN-13 treatment. B and D. Different
 54 capital letters indicate a significant effect of the time of stimulation with IGF1 (B) or FSH (D)
 55 whereas lower case letters indicate a significant effect of the APLN-13 treatment.

56

57 **Figure 5 : Effect of APLN-17 and APLN-13 on cell proliferation in absence or presence**

58 **of IGF1 in bovine granulosa cells.** Thymidine incorporation was determined in bovine GCs
 59 cultured for 24 h in serum-free medium in the presence or absence of APLN-17 (A, C) and
 60 APLN-13 (B, D) in basal state (A, B) or in response to IGF1 (10^{-8} M) (C, D) with or without
 61 ML221 as described in *Materials and Methods*. Results are expressed as thymidine
 62 incorporated in cpm. Results are representative of at least three independent experiments. The
 63 results are expressed as means \pm sem. Bars with different letters are significantly different (P
 64 < 0.05). **A and B.** Different capital letters indicate a significant effect of the concentration of
 65 APLN-17 (A) or APLN-13 (B) treatment whereas lower case letters indicate a significant
 66 effect of the ML221 treatment. **B and D.** Different capital letters indicate a significant effect
 67 of the treatment (APLN-17 (C), APLN-13 (D) or IGF1 (C and D) whereas lower case letters
 68 indicate a significant effect of the ML221 treatment.

69

70 **Figure 6 : Effect of APLN-13 (10^{-9} M) on (A) MAPK ERK1/2, (B) MAPK P38, (C) AKT**

71 **and (D) PRKA phosphorylation levels.** Bovine GC lysates were prepared from cells
 72 incubated with APLN-13 (10^{-9} M) for either 0, 1, 5, 10, 30 or 60 minutes. Lysates (50 μ g)
 73 were analyzed by immunoblotting using either (A) anti-phospho-MAPK ERK1/2, (B)
 74 phospho-MAPK P38, (C) phospho-AKT or (D) phospho-PRKA antibodies, and then with

75 anti-MAPK ERK2, AKT MAPK P38 and PRKA total protein antibodies. Representative blots
76 from three independent experiments are shown. Blots were quantified and the phospho-
77 protein to total protein ratios are shown. The results are presented as mean \pm SEM. Different
78 letters indicate significant differences ($P < 0.05$).

79

80 **Figure 7: Effect of APLN-13 (10^{-9} M) on the phosphorylation of (A) AKT, (B) MAPK**

81 **ERK1/2 ,(C) MAPK P38 and (D) PRKA in response to IGF1 in primary bovine**

82 **granulosa cells. (A-D):** Primary bovine granulosa cells were cultured in a media containing

83 serum, then cultured in serum-free media for 48 hours in the presence or absence of APLN-13

84 (10^{-9} M) , which were the same conditions used to measure progesterone production and then

85 stimulated with IGF1 (10^{-8} M) for 5 min. Cells were lysed and the lysates were directly

86 subjected to immunoblotting with antibodies against (A) phospho-AKT , (B) phospho-MAPK

87 ERK1/2, (C) phosphor-MAPK P38 or (D) anti-phospho-PRKA. The AKT, MAPK ERK1/2,

88 MAPK P38 and PRKA levels were evaluated by reprobng the membranes with antibodies for

89 total AKT, ERK2, MAPK P38 and PRKA, respectively. Representative blots from four

90 different cultures are shown. Blots were quantified and the phosphorylated protein to total

91 protein ratio is shown. The results are represented as mean \pm SEM. **E. Involvement of the**

92 **MAPK ERK1/2 signaling pathways in the effect of APLN-13 on the progesterone**

93 **secretion in the presence or in the absence of IGF1.** Overnight starved hGCs were

94 preincubated for one hour with either U0126 (10 μ M) or LY293004 (10 μ M) and then

95 cultured for 48 hours in serum-free media containing APLN-13 (10^{-9} M) in the absence or

96 presence of IGFI (10^{-8} M). (A) The culture medium was then collected and analyzed for

97 progesterone content. The results are expressed as protein concentration per well(ng/ml). The

98 results are presented as the mean \pm SEM of three independent cultures. Bars with different

99 letters are significantly different ($P < 0.05$).

100

101 **Figure 8: Involvement of the AKT signaling pathways in the effect of APLN-13 on the**
 102 **thymidine incorporation level in the presence or in the absence of IGF1.**

103 Thymidine incorporation was determined in overnight starved bovine granulosa cells
 104 preincubated for one hour with either U0126 (10 μ M), LY293004 (10 μ M), SB202190 (10
 105 μ M), or Compound C (10 μ M) and then stimulated for 24 h in serum-free media containing
 106 APLN-13 (10^{-9} M) in the absence or presence of IGF1 (10^{-8} M). Results are expressed as
 107 thymidine incorporated in cpm. Results are representative of at least three independent
 108 experiments. The results are expressed as means \pm sem.

109

110 **Figure 9: Effects of APLN-13 (A, B) and APLN-17 (C and D) on bovine oocyte nuclear**

111 **maturation. A.** Bovine oocytes were allowed to mature for 22 h in presence or absence of
 112 APLN-13 (10^{-9} M) (A) or APLN-17 (10^{-9} M) (C) with or without IGF1 (10^{-8} M) or FSH (10^{-8}
 113 8 M). The percentage of oocytes at the GV stage in the various conditions is shown. Different
 114 letters indicate significant differences with $P < 0.05$. The results are presented as mean \pm
 115 SEM of three independent experiments. At least 50 bovine oocytes for each set of conditions
 116 in each experiment were used. **B.** Bovine COCs were cultured for 22 h in maturation medium
 117 in presence or absence of APLN-13 (B) or APLN-17 (D) \pm IGF1 (10^{-8} M) or FSH (10^{-8} M).
 118 The culture medium was then collected, and its progesterone content was analyzed by Elisa as
 119 described in *Materials and Methods*. The results are expressed as ng/ml of 50 COC-
 120 equivalent cumulus cells. The results are mean \pm SEM for three independent experiments.
 121 Different letters indicate significant differences with $P < 0.05$.

122

123 **Figure 10: Effect of APLN-13 in absence or presence of IGF1 or FSH on MAPK ERK1/2**
 124 **(A) and PRKA (B) phosphorylation levels in bovine oocyte after nuclear maturation**

125 Bovine COCs were cultured for 22 h in maturation medium in presence or absence of APLN-
126 $13 \pm$ IGF1 (10^{-8} M) or FSH (10^{-8} M). COCs were then mechanically separated into oocyte and
127 cumulus cells. Denuded oocytes (50 oocytes per lane) were lysed and subjected to Western
128 blot analysis with antibodies against phospho-MAPK ERK1/2 (A) and phospho-PRKA (B).
129 Representative blots from three independent experiments are shown. Blots were quantified,
130 and the phosphorylated protein to total protein ratio is shown. Different letters indicate
131 significant differences with $P < 0.05$. The results are presented as mean \pm SEM.

132

133 **Supplemental Figure 1 : Effect of IGF1 and FSH on *APLN* and *APLNR* mRNA (A, B)**

134 **and protein (C, D) expression levels in primary bovine granulosa cells. (A, B) *APLN* and**

135 *APLNR* mRNA expression was measured by quantitative RT-PCR in primary bovine GCs

136 after 48 hours of stimulation with or without IGF1 (10^{-8} M) or FSH (10^{-8} M). The results

137 represent four cultures of GCs and are presented as mean \pm SEM. Different letters indicate

138 significant differences at $P < 0.05$. (C,D) Granulosa cell lysates were also subjected to

139 immunoblotting using antibodies raised against the APLN and APLNR protein. Equal protein

140 loading was verified by reprobing membranes with an anti-VCL antibody. Blots were

141 quantified and the APLN and APLNR to VCL ratio are shown. The results are expressed as

142 the mean \pm SEM of four independent cultures. Bars with different letters are significantly

143 different ($P < 0.05$).

144

145 **Supplemental Figure 2 : Picture of an immunoblot for APLN (A) and APLNR (B) in**

146 **primary bovine granulosa cells.**

147

148

149

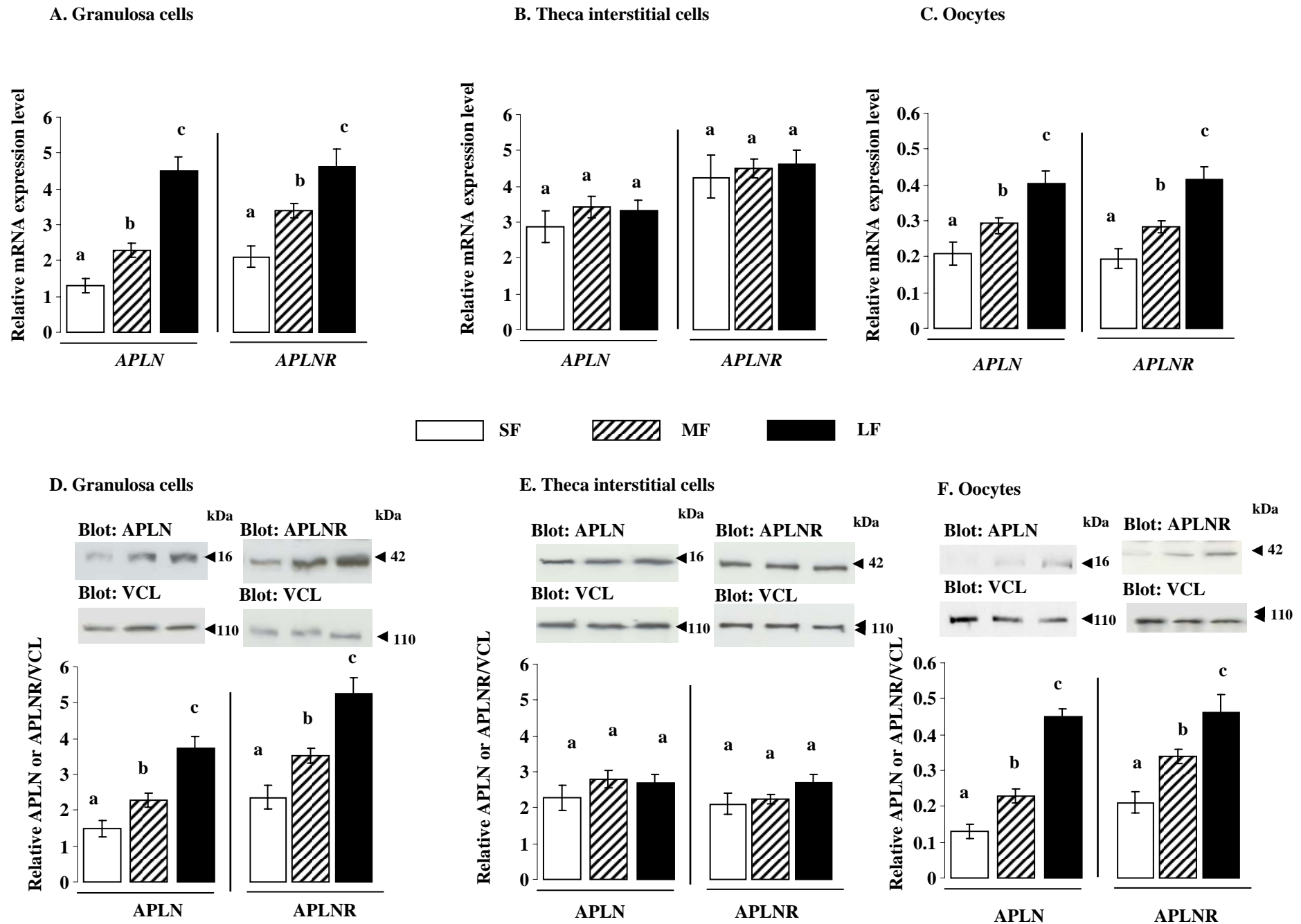


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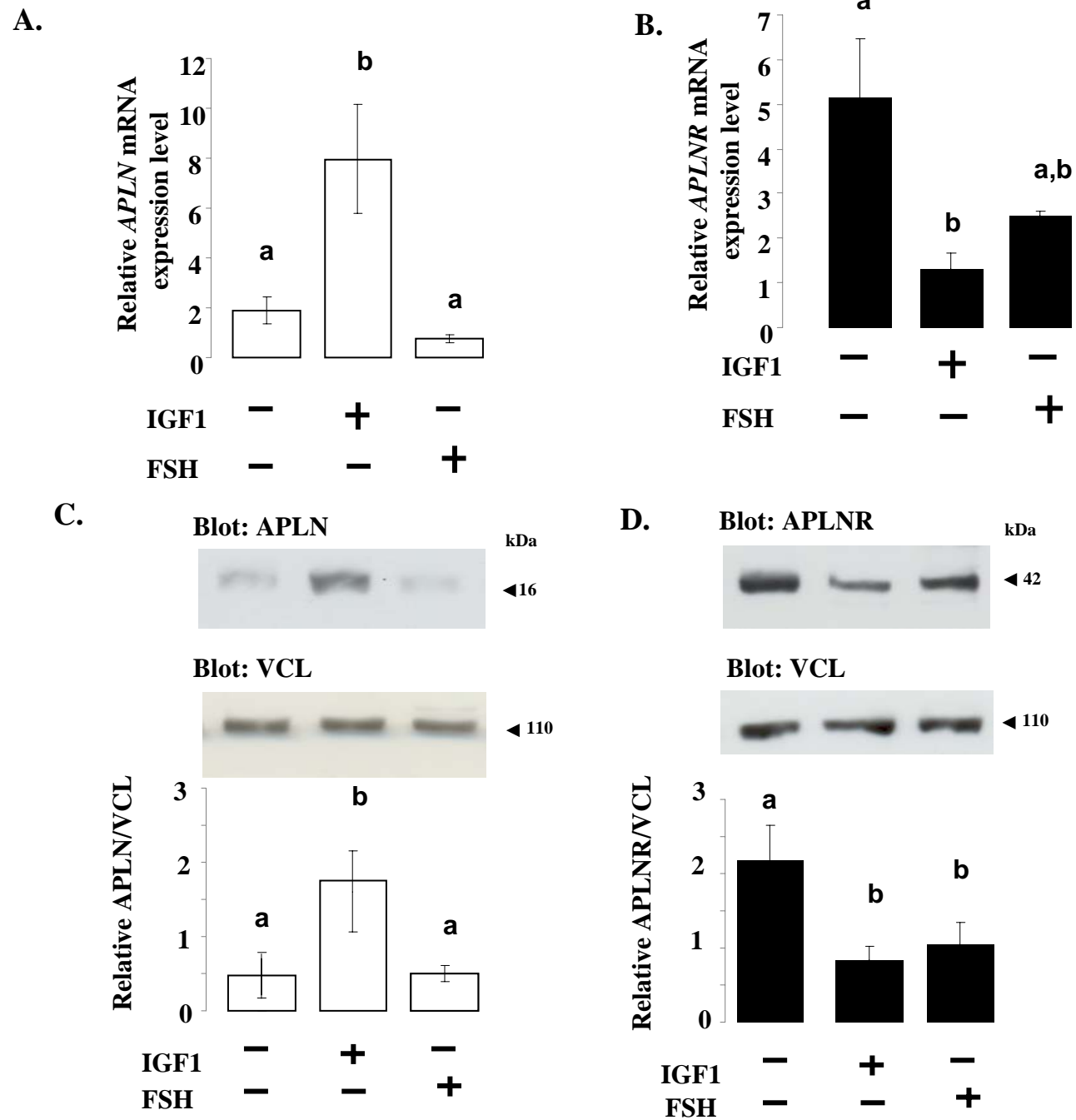


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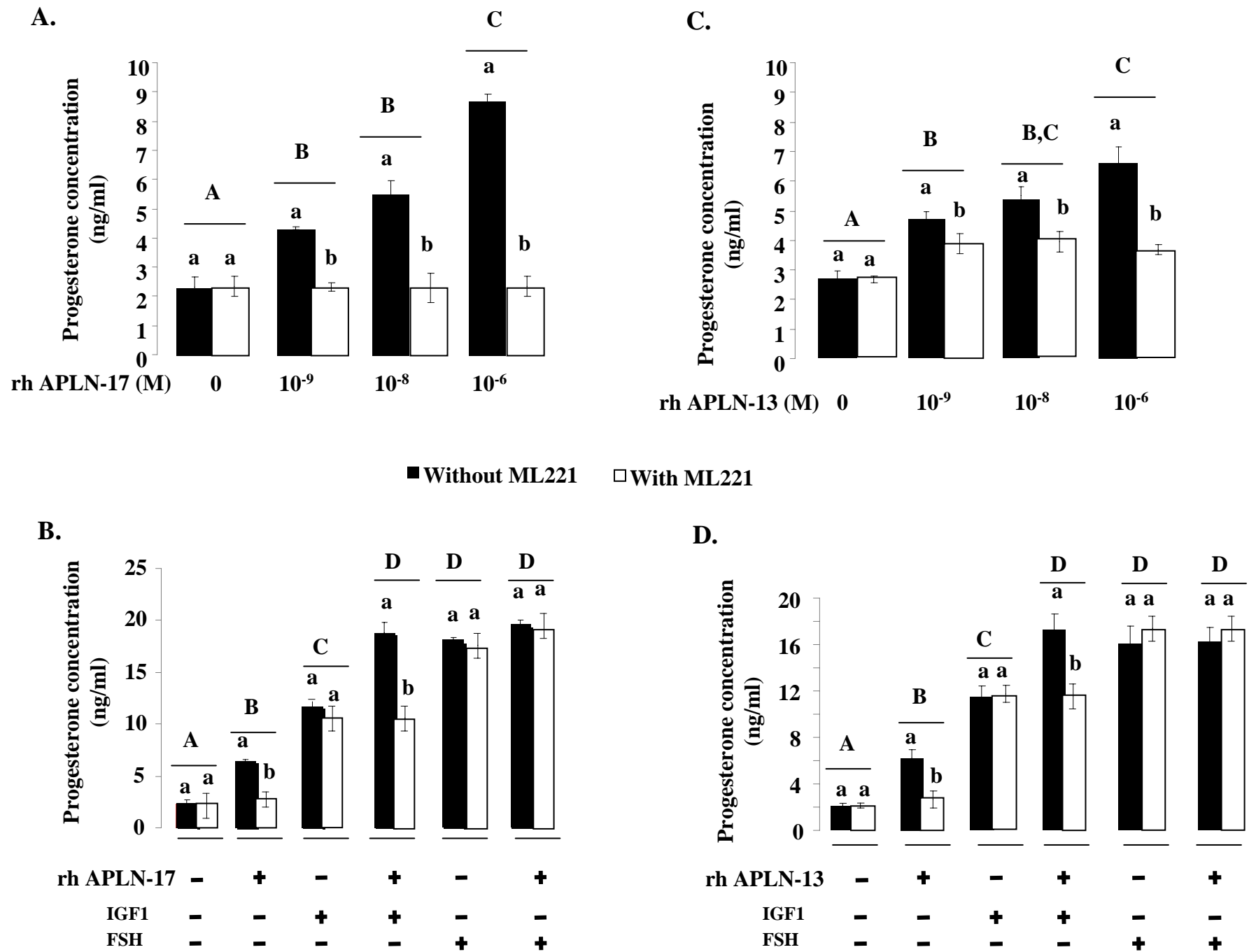


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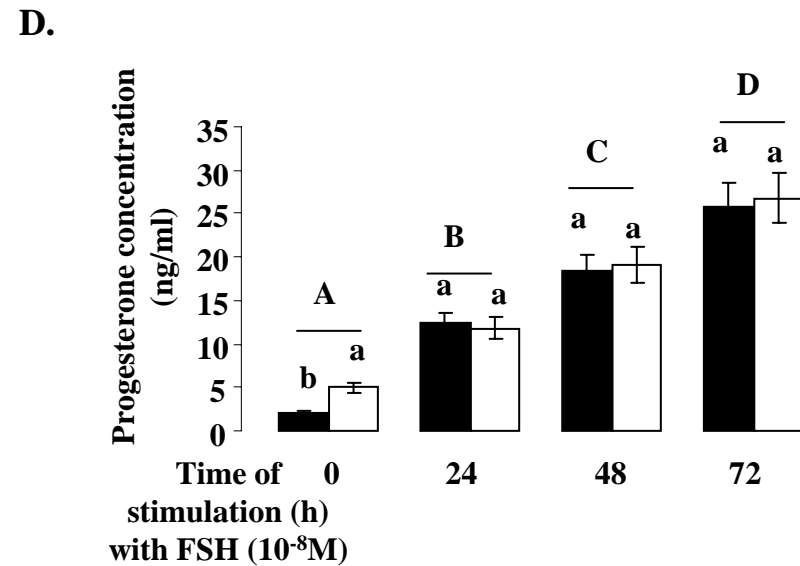
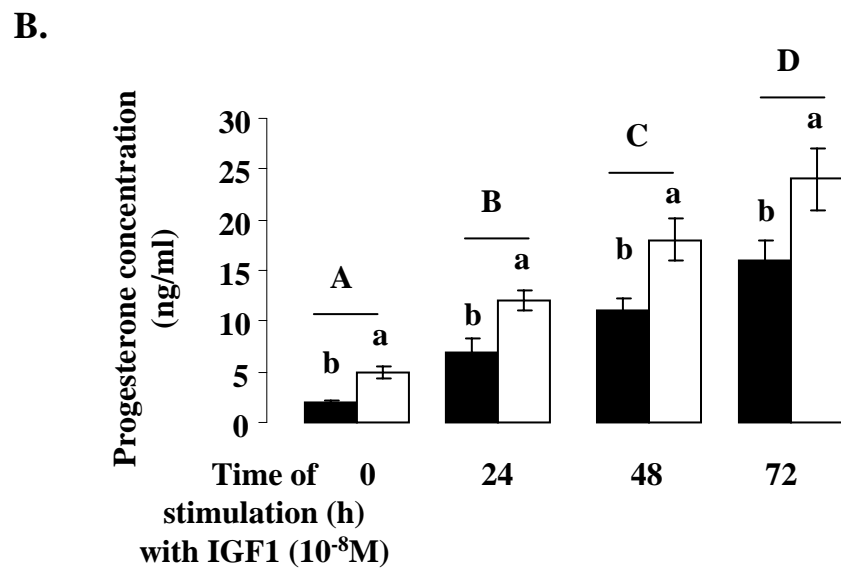
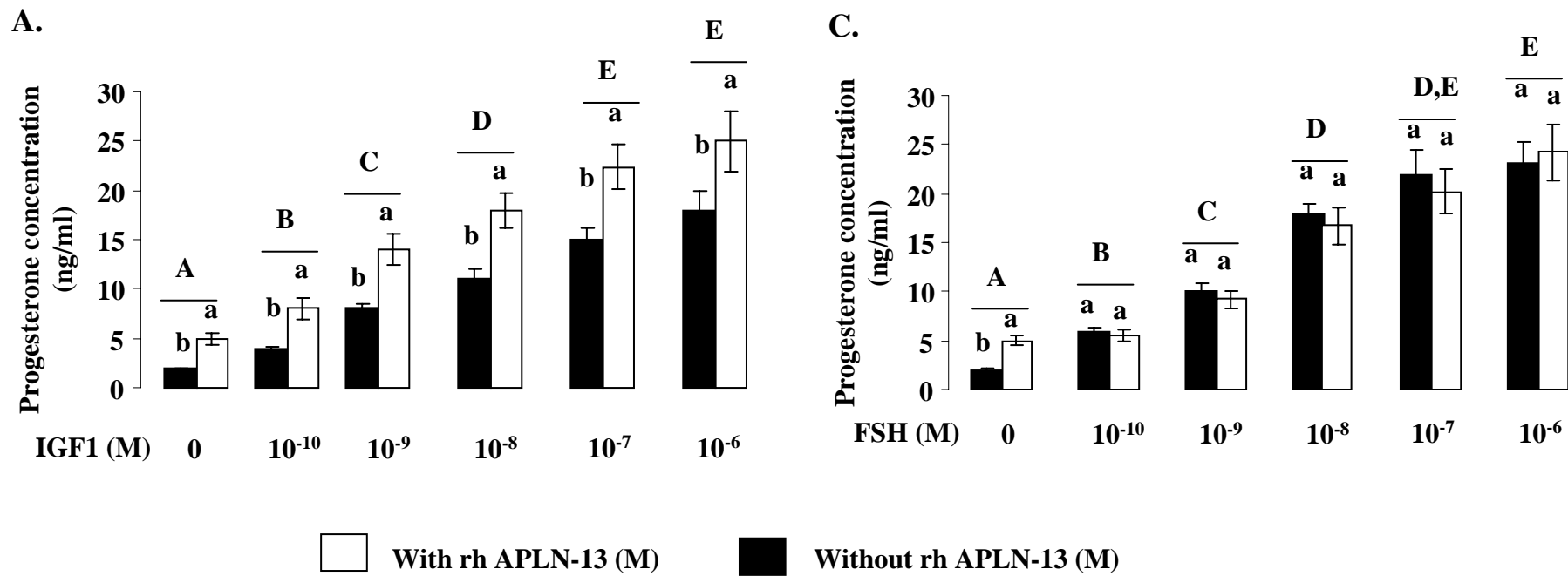


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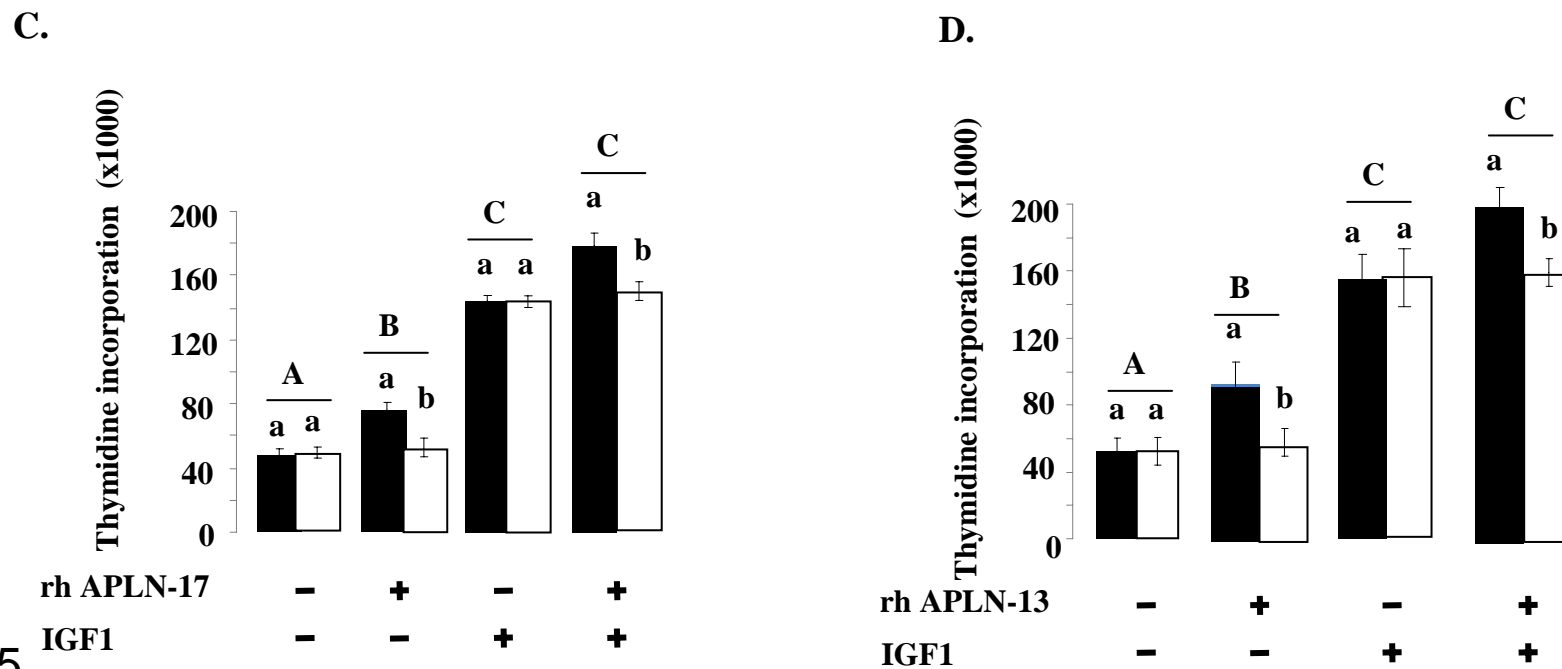
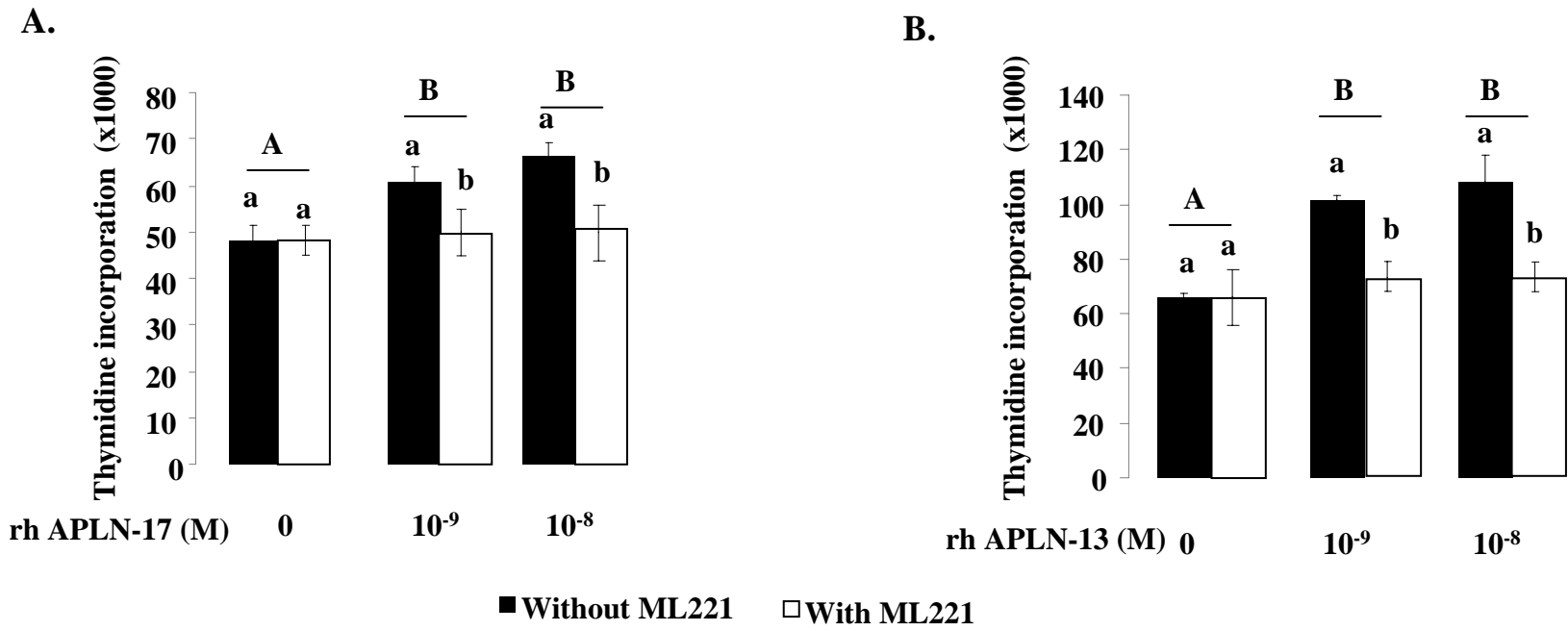


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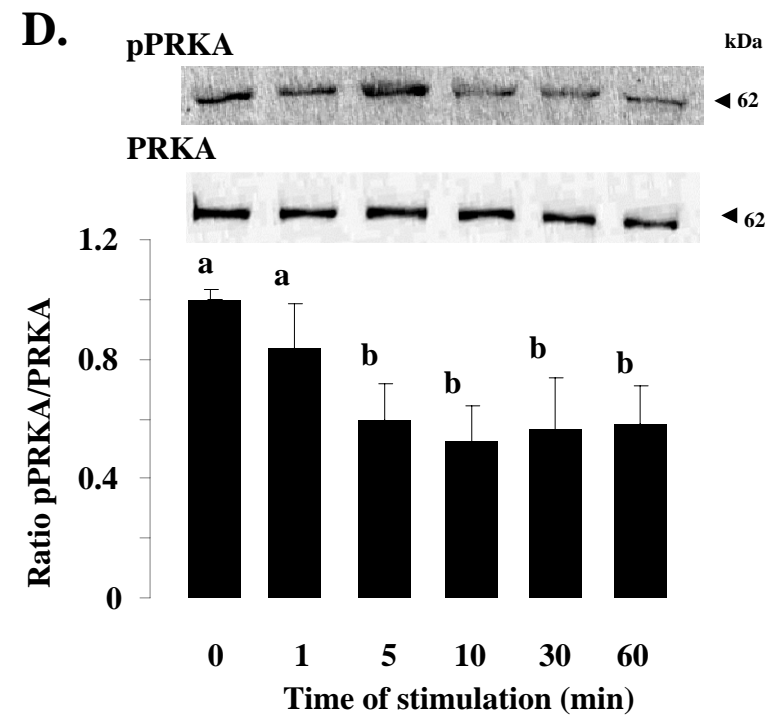
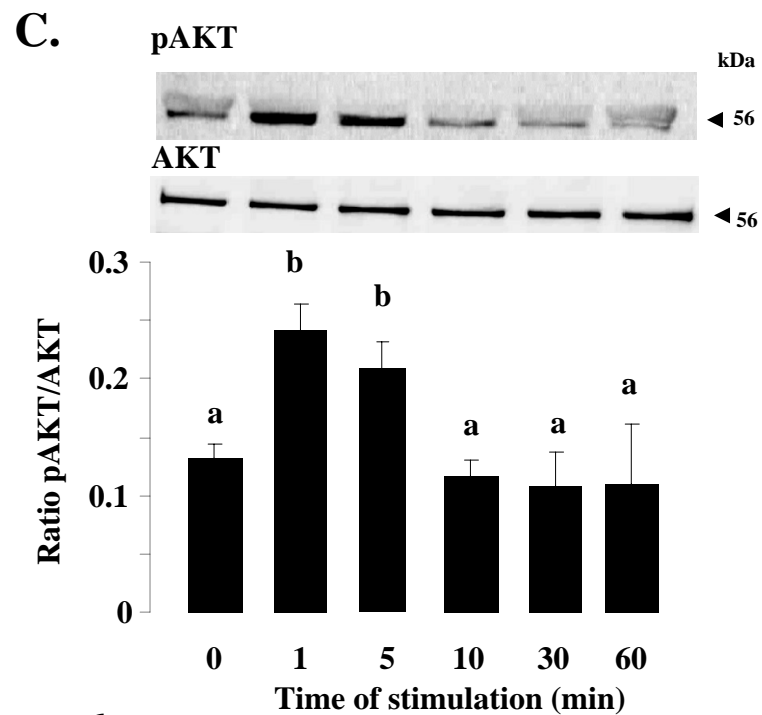
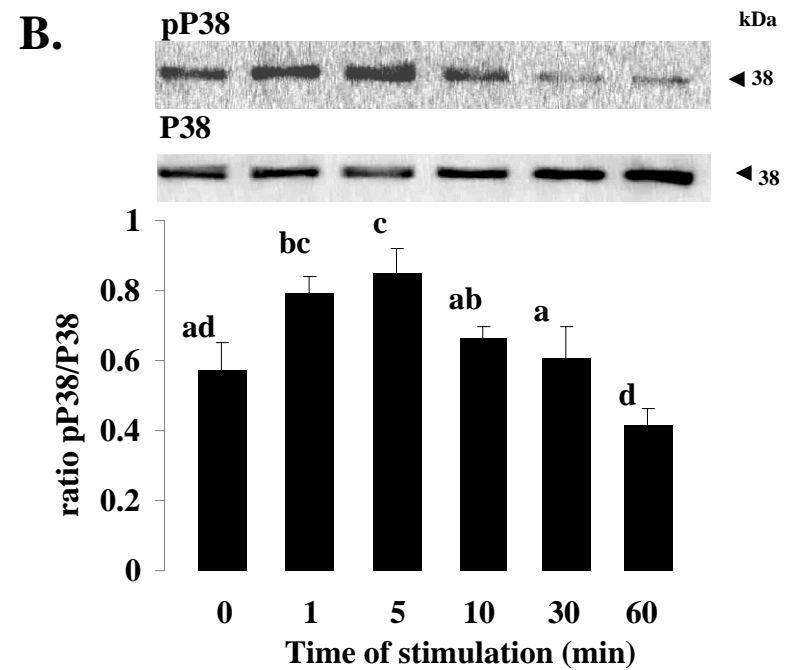
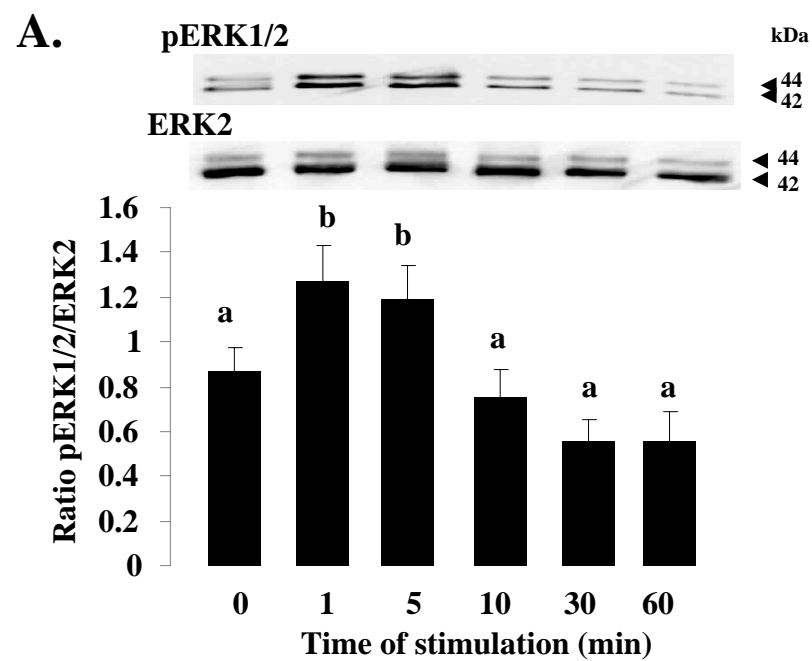


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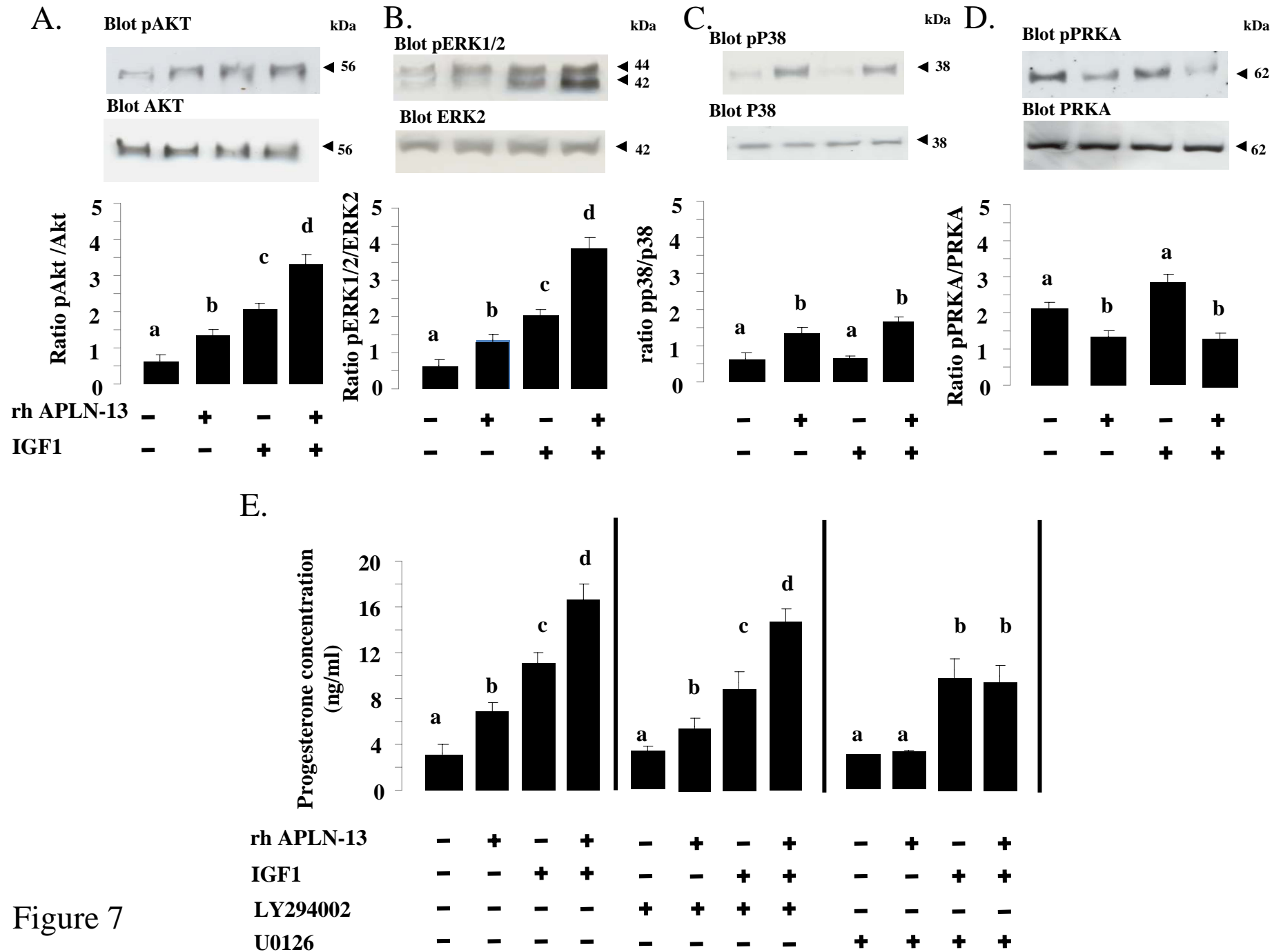


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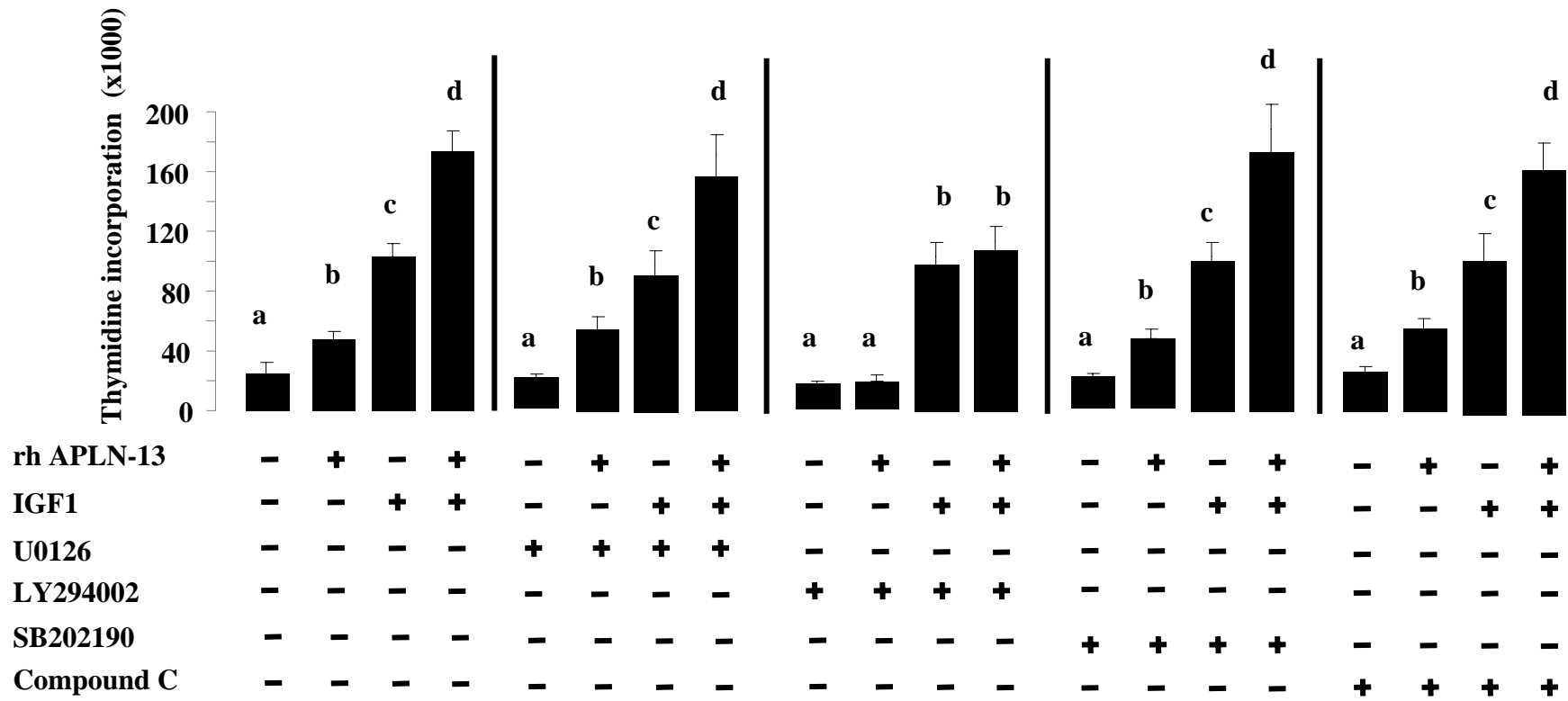
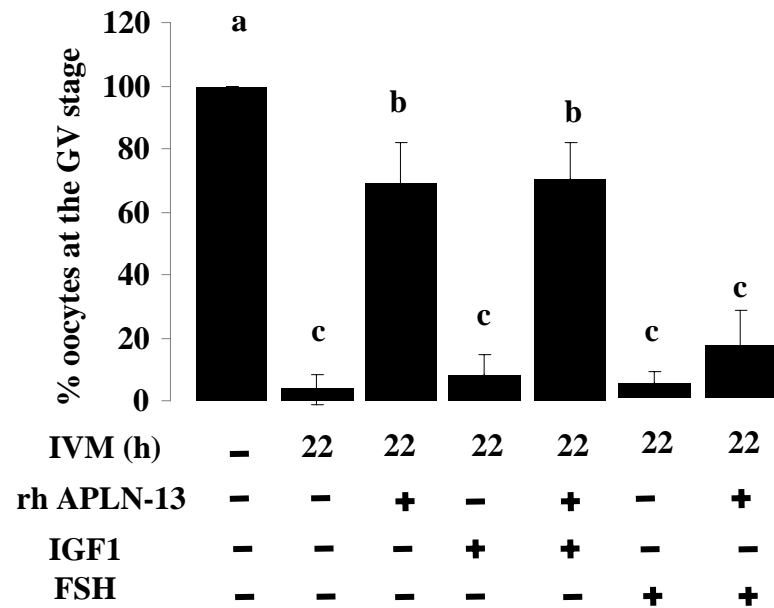
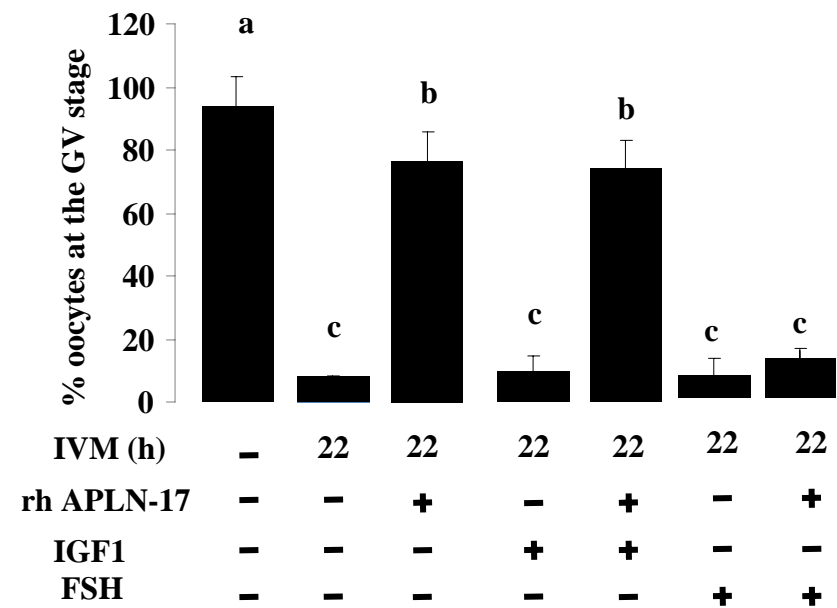


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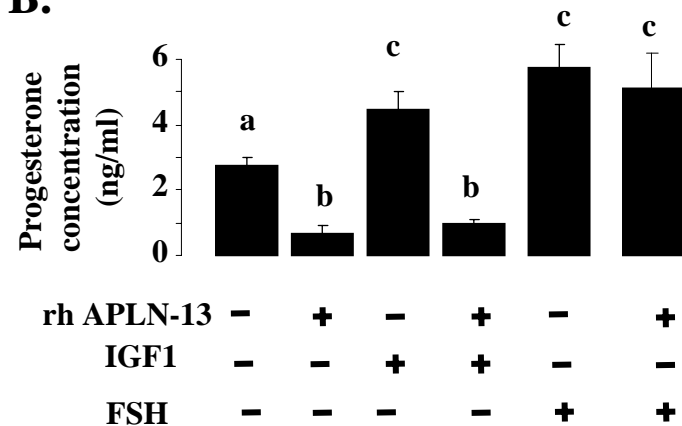
A.



C.



B.



D.

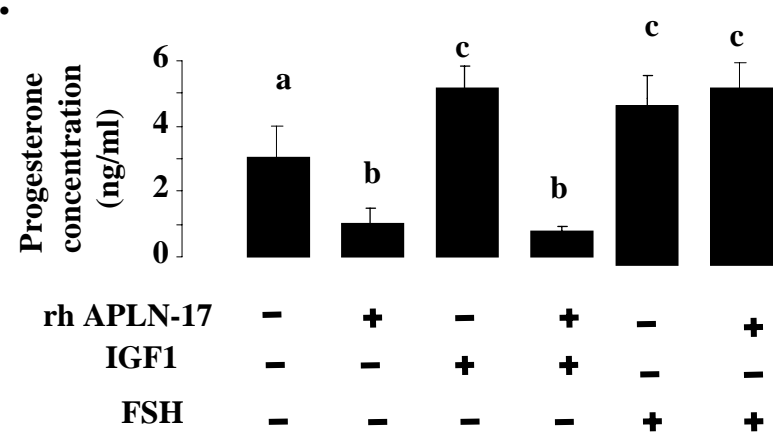
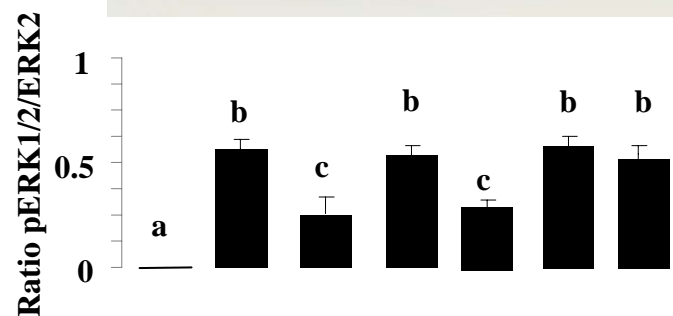
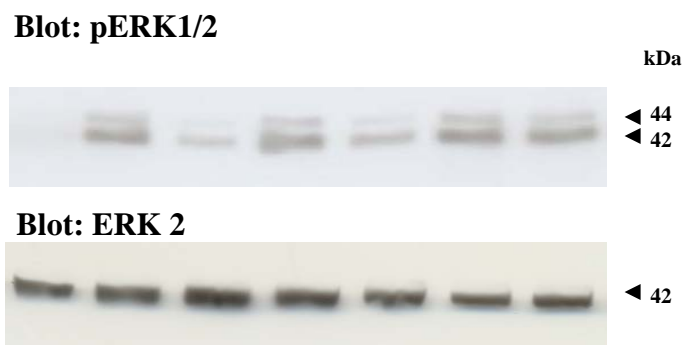


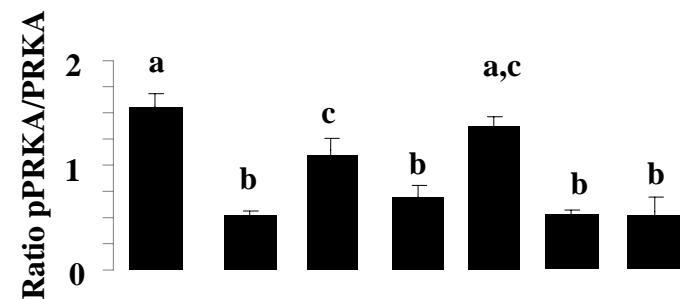
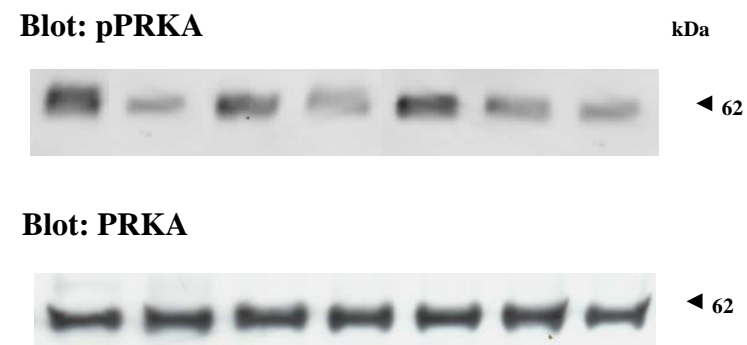
Figure 9

A.



IVM (h)	-	22	22	22	22	22	22
rh APLN-13	-	-	+	-	+	-	+
IGF1	-	-	-	+	+	-	-
FSH	-	-	-	-	-	+	+

B.



IVM (h)	-	22	22	22	22	22	22
rh APLN-13	-	-	+	-	+	-	+
IGF1	-	-	-	+	+	-	-
FSH	-	-	-	-	-	+	+

Figure 10