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► To cite this version:

Anthony Estienne, Belén Lahoz, Peggy Jarrier-Gaillard, Loys Bodin, José Folch, et al.. BMP15 regulates the inhibin/activin system independently of ovulation rate control in sheep. *Reproduction*, 2017, 153 (4), pp.395-404. 10.1530/REP-16-0507 . hal-01595250

HAL Id: hal-01595250

<https://hal.science/hal-01595250>

Submitted on 25 May 2020

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1 BMP15 regulates the inhibin/activin system independently of ovulation rate control in sheep

2

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15

16 Short title : Inhibin/activin system regulation by BMP15

17

18 **Abstract**

19 Polymorphisms in the gene encoding bone morphogenetic protein 15 (BMP15) have been
20 associated with multiple ovulations in sheep. As BMP15 regulates inhibin expression in
21 rodents, we assumed that the ovarian inhibin/activin system could mediate part of the effect of
22 *BMP15* mutations in the regulation of ovulation rate in sheep. To answer this question, we
23 have studied the effects of two natural loss-of-function mutations of *BMP15* on the expression
24 of components of this system. The *FecX^R* and the *FecX^{Gr}* mutations, when present
25 respectively in Rasa Aragonesa ewes at the heterozygous state and in Grivette ewes at the
26 homozygous state, were associated with a two-fold increase in ovulation rate. There were only
27 small differences between mutant and wild-type ewes for mRNA expression of *INHA*,
28 *INHBA*, *ACVR1B*, *ACVR2A*, *FST* or *TGFBR3* in granulosa cells, and Inhibin A or Activin A
29 concentrations in follicular fluid. Moreover, the effects of mutations differed between breeds.
30 In cultures of granulosa cells from wild-type ewes, BMP15, acting alone or in synergy with
31 GDF9, stimulated *INHA*, *INHBA* and *FST* expression, but inhibited the expression of
32 *TGFBR3*. Activin A did not affect *INHBA* expression, but inhibited also the expression of
33 *ACVR2A*. The complexity of the inhibin/activin system, including positive and antagonistic
34 elements, and the differential regulation of these elements by BMP15 and activin can explain
35 that the effects of *BMP15* mutations differ when present in different genetic backgrounds. In
36 conclusion, the ovarian inhibin/activin system is unlikely to participate in the increase of
37 ovulation rate associated with *BMP15* mutations in sheep.

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Comment citer ce document :

Estienne, A., Lahoz, B., Jarrier, P., Bodin, L., Folch, J., Alabart, J. L., Fabre, S.,
Monniaux, D. (2017). BMP15 regulates the inhibin/activin system independently of ovulation rate control
in sheep. *Reproduction*, 153 (4), 395-404. DOI : 10.1530/REP-16-0507

39 Introduction

40 Increasing evidence support the involvement of cytokines belonging to the
41 transforming growth factor beta (TGF β) family in the control of ovulation number at each
42 sexual cycle (ovulation rate) in adult females. In particular, the bone morphogenetic proteins
43 BMP15 (bone morphogenetic protein 15) and GDF9 (growth differentiation factor 9), known
44 to be specifically expressed in the oocyte (McGrath *et al.* 1995; Dube *et al.* 1998), could play
45 a key role in this process. In sheep, heterozygous carriers of various missense mutations
46 affecting *BMP15* or *GDF9*, or carriers of a partial loss-of-function mutation in their receptor
47 *BMPRII*, exhibit systematically increased ovulation rates and litter sizes (reviews:
48 (Montgomery *et al.* 2001; Galloway *et al.* 2002; McNatty *et al.* 2003; Fabre *et al.* 2006;
49 Persani *et al.* 2014). Polymorphisms in *GDF9*, *BMPRII* and *SMAD3* (encoding a
50 BMP15/GDF9 signaling factor) have also been shown to occur among human cohorts with a
51 propensity for dizygotic twins (Palmer *et al.* 2006; Hoekstra *et al.* 2008; Luong *et al.* 2011;
52 Mbarek *et al.* 2016) and the presence of non-synonymous substitutions in *BMP15* or *GDF9*
53 genes has been recently associated with twinning in callitrichine primates (Harris *et al.* 2014).
54 From a phylogenetic analysis comparing mono- and poly-ovulating mammalian species, high
55 variations have been observed in specific areas of BMP15 and GDF9, able to modify the
56 biological activity of the proteins in poly-ovulating mammals (Monestier *et al.* 2014).

57 The mechanism by which BMP15 and GDF9 control the ovulation rate is not fully
58 understood. BMP15, acting alone or in synergy with GDF9, can modulate follicle growth and
59 maturation by controlling granulosa cell proliferation and their responsiveness to FSH
60 (Otsuka *et al.* 2000; Otsuka *et al.* 2001; Moore *et al.* 2003; McNatty *et al.* 2005b; Moore &
61 Shimasaki 2005). Recently, the anti-Müllerian hormone (AMH), another member of the
62 TGF β family known to importantly modulate FSH sensitivity (Durlinger *et al.* 2001; Visser &
63 Themmen 2005; Knight & Glister 2006; Visser & Themmen 2014), has also been proposed to

64 participate in this mechanism. Indeed, BMP15 enhances the expression of AMH and its
65 specific receptor AMHR2 in the granulosa cells of sheep antral follicles, and hyperprolific
66 ewes carrying a loss-of-function mutation in *BMP15* or its receptor *BMPRIIB* show
67 respectively impaired expression of AMHR2 or AMH (Estienne *et al.* 2015; Pierre *et al.*
68 2016). It is suggested that low BMP15 and low AMH signaling in follicles of these mutant
69 ewes contribute to sensitizing granulosa cells to FSH, thereby promoting the selection and
70 maintenance of follicles during the follicular phase, and thus enhancing the ovulation rate.

71 Inhibins and activins, other members of the TGF β family, could also be involved in
72 the mechanisms leading to multiple ovulations. Inhibins and activins are produced by the
73 granulosa cells of growing follicles and known to act in an opposite way on their target cells.
74 Activins are importantly implicated in follicular growth, acting in an intra-ovarian way by
75 promoting granulosa cell proliferation, enhancing FSHR (follicle stimulating hormone
76 receptor) and CYP19A1 (cytochrome P450 family 19 subfamily A member 1, also known as
77 aromatase) expression, and inhibiting follicle atresia and luteinization, whereas inhibins
78 oppose the activin signaling, thus modulating these processes (Knight *et al.* 2012). Inhibins
79 are secreted by the largest follicles during terminal follicular development and are important
80 inhibitors of FSH secretion by pituitary gonadotrophs. In various animal species,
81 immunization against inhibin induces multiple ovulations and an increase in pituitary
82 secretion of FSH appears to be the main mechanism by which the growth of additional
83 follicles is stimulated (Henderson *et al.* 1984; Forage *et al.* 1987; Hillard *et al.* 1995;
84 Drummond *et al.* 2004; Yan *et al.* 2015). However, immunization against inhibin is not
85 always accompanied by the expected rise in plasma FSH, and intra-ovarian effects of
86 neutralizing endogenous inhibin bioactivity might also be involved in the stimulation of
87 follicular development (Tannetta *et al.* 1998). Interestingly, the specific intra-ovarian
88 glycosylation of the inhibin alpha and beta A subunits is associated with multiple ovulations

89 and high prolificacy in ewes carrying the *FecL^L* mutation which induces an ectopic expression
90 of the *B4GALNT2* (*beta-1,4-N-acetyl-galactosaminyl transferase 2*) gene within the ovary
91 (Drouilhet *et al.* 2013).

92 We assumed that the ovarian inhibin/activin system could mediate part of the effect of
93 *BMP15* mutations in the regulation of ovulation rate in sheep. This assumption is supported
94 by the findings that BMP15 can activate *in vitro* the transcription of genes encoding inhibin
95 subunits and follistatin (the specific activin binding protein) in mouse granulosa cells (Li *et al.*
96 2009) and cooperates with GDF9 in enhancing immunoreactive inhibin production in rat
97 granulosa cells (McNatty *et al.* 2005a). To answer this question, we have analyzed the *in vivo*
98 effects of two natural loss-of-function mutations of *BMP15* in two ovine breeds on the
99 expression of components of the inhibin/activin system in relationship with the hyperprolific
100 phenotype of mutant ewes. We took into consideration various components of this system,
101 including the INHA and INHBA subunits, the activin signaling receptors ACVR1B and
102 ACVR2A and the known antagonists of activin, which are follistatin (FST) and the TGF β
103 type 3 receptor (TGFBR3, also known as betaglycan, a co-receptor of inhibin, able to block
104 activin signaling) (Walton *et al.* 2012). In addition, we studied the acute *in vitro* effects of
105 BMP15 on the mRNA expression of these components in ovine granulosa cells, in
106 comparison with those of other factors of the TGF β family such as BMP4, GDF9 and Activin
107 A.

108

109 **Materials and Methods**

110 *Animals*

111 For *in vivo* expression studies, granulosa cells were recovered from hyperprolific ewes
112 carrying mutations in the *BMP15* gene and their corresponding non-carrier ewes of the same
113 breed. The *BMP15*^{W154NFsX55}/*FecX*^R mutation is a 17 bp deletion creating a premature stop

114 codon in the *BMP15* pro-region; it causes hyperprolificacy in the Spanish Rasa Aragonesa
115 breed when the mutation is present in ewes at the heterozygous state and sterility when
116 present at the homozygous state (Martinez-Royo *et al.* 2008). The *BMP15*^{T317I}/*FecX*^{Gr}
117 mutation is a substitution of a threonine for an isoleucine which affects the hydrophobicity of
118 the protein and impairs its signaling activity; it causes hyperprolificacy in the French Grivette
119 breed when the mutation is present at the heterozygous state and even more at the
120 homozygous state (Demars *et al.* 2013). In this study, 6 Rasa Aragonesa ewes heterozygous
121 carriers of the *FecX*^R mutation (*R/+*; ovulation rate = 1.89 ± 0.16), 6 Rasa Aragonesa non-
122 carrier ewes (*+/+*; ovulation rate = 1.00), 5 Grivette ewes homozygous carriers of the *FecX*^{Gr}
123 mutation (*Gr/Gr*; ovulation rate = 4.88 ± 0.55) and 5 Grivette non-carrier ewes (*+/+*;
124 ovulation rate = 2.29 ± 0.20) were used after genotyping.

125 All animals were treated during their seasonal period of reproduction with intravaginal
126 progestagen sponges (fluogestone acetate, 20 mg; Intervet, Angers, France) for 14 days to
127 synchronize estrus, before slaughtering of the ewes 36 h after sponge removal, corresponding
128 to the follicular phase of the cycle. Ovaries from all genotypes were recovered in a local
129 slaughterhouse at Zaragoza (Spain) and Toulouse (France), for the Rasa Aragonesa and
130 Grivette breeds, respectively. All procedures were approved by the Agricultural and Scientific
131 Research Government Committees and local ethical committees (Approval numbers C37-175-
132 2 in Nouzilly, C31-429-01 in Toulouse and - CITA 2011-08 in Zaragoza) in accordance with
133 the guidelines for Care and Use of Agricultural Animals in Agricultural Research and
134 Teaching.

135 *Collection and culture of granulosa cells*

136 Collected ovaries were finely dissected to isolate all the antral follicles larger than 1
137 mm in diameter. For each animal, the follicles were then classified according to their size and
138 genotype. For the evaluation of *in vivo* gene expression in Rasa Aragonesa and Grivette ewes,

139 two follicular classes were defined, small (1–3 mm) and large (> or equal to 5 mm) follicles,
140 corresponding to follicle growth and dominance stages, respectively. Follicular fluid and
141 granulosa cells were recovered from small and large follicles as described (Estienne *et al.*
142 2015). For small follicles, follicular fluid and granulosa cells were pooled within animal. The
143 volumes of follicular fluid recovered by follicular puncture from each animal were at least 50
144 µl for individual large follicles and 5 µl for pooled small follicles. Follicular fluids and
145 granulosa cells were stored at -80°C for further analysis.

146 For culture experiments, ovaries were recovered from ewes of various breeds wild-
147 type at the two *BMP15* loci after slaughtering in a local slaughterhouse at Nouzilly (France).
148 Granulosa cells from 1-3 mm follicles were cultured in serum-free conditions in McCoy's 5a
149 medium (Sigma, L'Isle d'Abeau Chesnes, France) containing insulin (100 ng/ml) according to
150 a previously described method (Campbell 1996). Cells were seeded at 10⁵ viable cells/well in
151 96-well plates with and without recombinant human BMP4, human BMP15, mouse GDF9 or
152 human/mouse/rat Activin A (50 ng/ml each; R&D System Europe, Lille, France), each
153 treatment being tested in 12 replicate wells. After 48 h of culture, cells were recovered using
154 accutase (Sigma), then pooled according to treatment, centrifuged, and cell pellets were stored
155 at -80°C for further RNA extraction. In total, 15 independent culture experiments were
156 performed.

157 *RNA extraction and quantitative real-time PCR*

158 Samples of granulosa cell recovered *in vivo* and after culture were analyzed for mRNA
159 content of genes encoding components of the inhibin/activin system (*INHA*, *INHBA*,
160 *ACVR1B*, *ACVR2A*, *FST*, *TGFBR3*). For *in vivo* gene expression studies, the stage of
161 follicular maturation and health was assessed using classic markers of differentiation
162 (*CYP19A1*) and atresia (*IGFBP5*) in ovine granulosa cells (Besnard *et al.* 1996; Logan *et al.*
163 2002). Total RNA was extracted with a Nucleospin RNA II kit (Macherey-Nagel, Hoerd,

164 France) according to the manufacturer's protocol. Then, 1 µg of RNA was reverse-
165 transcribed, and quantitative real-time PCR reactions were run using SYBR Green Supermix
166 on an iCycler iQ multicolor detection system (Bio-Rad, Marnes la Coquette, France) as
167 previously described (Monniaux *et al.* 2008). The specific primer sequences used for
168 amplification are listed in Table 1. For each primer pair, amplification efficiency (E) was
169 measured as described (Monniaux *et al.* 2008). The cycle threshold (Ct) of the target gene was
170 compared with that of the *RPL19* internal reference gene encoding a ubiquitous ribosomal
171 protein, and the mRNA relative level was estimated by the ratio $R = 100 \times [E_{RPL19}^{Ct}$
172 $(RPL19)/E_{target}^{Ct(target)}]$. *RPL19* is known to be easily detectable and not regulated during ovine
173 follicular growth (Bonnet *et al.* 2011) and it has been used and validated as a reference gene
174 in the granulosa cells of sheep follicles in previous studies (Drouilhet *et al.* 2013; Estienne *et*
175 *al.* 2015; Pierre *et al.* 2016).

176 *Inhibin and activin assays*

177 Inhibin A concentration in follicular fluid was measured using the Active Inhibin A
178 ELISA kit (Beckman Coulter France) according to the manufacturer's protocol. The use of the
179 Active Inhibin A ELISA kit in sheep has been validated by studying serial dilutions of
180 different ovine plasma and follicular fluid samples in steer plasma. Results showed that the
181 dilution curves were linear and parallel to the standard curve (Supplementary Figure 1A). In
182 the present study, Inhibin A concentrations were determined in 30 µl aliquots of follicular
183 fluid diluted at 1/100 and 1/1000 for small and large follicles, respectively. In our working
184 conditions, the limit of detection of the assay was found to be 0.5 pg/well and the intra- and
185 inter-assays coefficients of variation were lower than 5%.

186 Activin A concentration in follicular fluid was measured using the Human/Mouse/Rat
187 Activin A Quantikine ELISA kit (R&D Systems Europe LTD) according to the manufacturer's
188 protocol. The use of the Human/Mouse/Rat Activin A Quantikine ELISA kit in sheep has been

189 validated by studying serial dilutions of different ovine plasma and follicular fluid samples in
190 steer plasma. Results showed that the dilution curves were linear and parallel to the standard
191 curve (Supplementary Figure 1B). In the present study, Activin A concentrations were
192 determined in 100 μ l aliquots of follicular fluid diluted at 1/1000 and 1/10000 for small and
193 large follicles, respectively. In our working conditions, the limit of detection of the assay was
194 found to be 1.5 pg/well and the intra- and inter-assays coefficients of variation were lower
195 than 5% and 7%, respectively.

196 *Statistics*

197 All data are presented as mean \pm SEM. Data were analyzed using the GraphPad Prism
198 6 Software (GraphPad Software Inc., La Jolla, CA, USA). In the wild-type (+/+) ewes,
199 comparisons of follicle numbers and sizes between the Rasa Aragonesa and the Grivette
200 breeds were made using Mann Whitney test, and comparisons of gene expression and
201 hormonal concentrations in follicular fluid between breeds and follicular size classes were
202 made using two-way ANOVA. Within each ovine breed, gene expression and hormonal
203 concentrations were compared between follicular classes and genotypes using two-way
204 ANOVA. The ANOVA analyses were followed by Fisher's protected Least Significant
205 Difference (LSD) test for multiple comparisons. For granulosa cell culture experiments, data
206 were analyzed using repeated measures one-way ANOVA with the Greenhouse-Geisser
207 correction of the degrees of freedom. For all analyses, a probability of at least 0.05 was
208 required for significance.

209

210 **Results**

211 *Follicular development in the Rasa Aragonesa and Grivette breeds and effects of*
212 *BMP15 mutations*

213 There was no difference between the Rasa Aragonesa and Grivette breeds for the
214 numbers and sizes of follicles present in the ovaries of the wild-type (+/+) ewes, but follicular
215 development was affected by the presence of *BMP15* mutations in both breeds. In the Rasa
216 Aragonesa breed, the number of large dominant follicles (> or equal to 5 mm in the follicular
217 phase of the estrous cycle) was higher in the prolific *R/+* than in the non-prolific +/+ ewes
218 ($p < 0.05$) and the mean size of these large follicles was smaller ($p < 0.01$, Table 2). In the
219 Grivette breed, the number of large follicles was not statistically different between genotypes,
220 although the mean size of the large follicles was smaller in the prolific *Gr/Gr* ewes than in the
221 +/+ ewes ($p < 0.001$, Table 2). In both breeds, the number of small (1-3 mm) follicles was
222 similar between genotypes.

223 In +/+ ewes, there was no difference between the Rasa Aragonesa and Grivette breeds
224 for *CYP19A1* (a marker of follicle maturation) and *IGFBP5* (a marker of follicle atresia)
225 expression in granulosa cells. As expected, in both breeds and all genotypes, *CYP19A1*
226 mRNA levels were strongly increased in the granulosa cells of the large follicles, compared to
227 the small ones ($p < 0.001$ for all comparisons, Figure 1). This increase in granulosa cell
228 differentiation was associated with a lower expression of the atresia marker *IGFBP5* in the
229 large follicles (Rasa Aragonesa breed: $p < 0.001$ for both +/+ and *R/+* follicles; Grivette breed:
230 $p < 0.05$ for +/+ follicles and $p < 0.01$ for *Gr/Gr* follicles). There was no difference between
231 genotypes, except a 1.9-fold lower *CYP19A1* expression in *R/+*, compared to +/+ large
232 follicles, in the Rasa Aragonesa breed ($p < 0.05$, Figure 1).

233 *Ovarian expression of Inhibin A and Activin A in the Rasa Aragonesa and Grivette*
234 *breeds and effects of BMP15 mutations*

235 The mRNA levels of *INHHA* and *INHBA* were higher ($p < 0.001$ for both genes) in +/+
236 granulosa cells from large follicles of the Grivette, compared to the Rasa Aragonesa breed. In
237 both breeds and all genotypes, *INHHA* and *INHBA* mRNA levels were strongly increased in the

238 granulosa cells of the large follicles, compared to the small ones ($p < 0.001$ for both genes and
239 all comparisons, Figure 2). Within each breed, there was no difference between genotypes for
240 *INHA* and *INHBA* mRNA levels.

241 The intra-follicular concentrations of Inhibin A and Activin A were both higher
242 ($p < 0.001$ and $p < 0.01$, respectively) in $+/+$ large follicles of the Grivette, compared to the
243 Rasa Aragonesa breed, but the Activin A / Inhibin A concentration ratio was similar between
244 breeds. In both breeds and all genotypes, the intra-follicular concentrations of Inhibin A were
245 higher in the large follicles, compared to the small ones ($p < 0.001$ for all comparisons) and,
246 except for the Rasa Aragonesa $+/+$ ewes, the intra-follicular concentrations of Activin A were
247 also significantly higher in the large follicles ($p < 0.05$ for all comparisons, Figure 3). In both
248 breeds and all genotypes, the Activin A / Inhibin A concentration ratio was lower in the large
249 follicles, compared to the small ones ($p < 0.001$ for all comparisons). No significant differences
250 were found between genotypes, except a 2.3-fold higher concentration of Inhibin A in $R/+$,
251 compared to $+/+$ large follicles, in the Rasa Aragonesa breed ($p < 0.05$, Figure 3).

252 *Ovarian expression of activin receptors and activin/inhibin binding proteins in the*
253 *Rasa Aragonesa and Grivette breeds and effects of BMP15 mutations*

254 The mRNA levels of *ACVR1B* and *ACVR2A* were higher in $+/+$ granulosa cells from
255 large follicles ($p < 0.001$ for both genes) and small follicles ($p < 0.05$ and $p < 0.001$ for *ACVR1B*
256 and *ACVR2A*, respectively) of the Grivette, compared to the Rasa Aragonesa breed. In the
257 Grivette breed, *ACVR1B* and *ACVR2A* mRNA levels were higher in the granulosa cells of the
258 large follicles, compared to the small ones for both genotypes (*ACVR1B* and *ACVR2A*:
259 $p < 0.001$ for $+/+$ follicles and $p < 0.05$ for *Gr/Gr* follicles, Figure 4), but there was no
260 significant difference between follicular sizes in the Rasa Aragonesa breed, except higher
261 *ACVR2A* mRNA levels in the large $R/+$ follicles, compared to the small ones of the same
262 genotype ($p < 0.01$). In the Grivette breed, *ACVR1B* mRNA levels were 1.9-fold lower

263 ($p < 0.01$) and *ACVR2A* mRNA levels were 1.6-fold lower ($p < 0.01$) in *Gr/Gr*, compared to +/+
 264 large follicles (Figure 4), but no difference between genotypes was observed in the Rasa
 265 Aragonesa breed.

266 The mRNA levels of *FST* and *TGFBR3* were lower ($p < 0.05$ and $p < 0.01$, respectively)
 267 in +/+ granulosa cells from large follicles of the Grivette, compared to the Rasa Aragonesa
 268 breed. In both breeds and all genotypes, *FST* and *TGFBR3* mRNA levels were increased in the
 269 granulosa cells of the large follicles, compared to the small ones (*FST*: $p < 0.001$ for both
 270 breeds and all genotypes, *TGFBR3*: $p < 0.001$ for both genotypes in Rasa Aragonesa breed,
 271 $p < 0.001$ for +/+ and $p < 0.01$ for *Gr/Gr* genotypes in Grivette breed, Figure 5). *FST* mRNA
 272 levels were 1.6-fold lower ($p < 0.05$) in *R/+*, compared to +/+ small follicles in the Rasa
 273 Aragonesa breed (Figure 5), but no difference in *FST* expression was observed between
 274 genotypes in the Grivette breed. *TGFBR3* mRNA levels were 1.4-fold lower ($p < 0.05$) in
 275 *Gr/Gr*, compared to +/+ large follicles in the Grivette breed (Figure 5), but no difference
 276 between genotypes was observed in the Rasa Aragonesa breed.

277 *Regulation of the components of the inhibin/activin system by BMP15 and Activin A in*
 278 *ovine granulosa cells in vitro*

279 The small (< 3-fold changes), or non-existent differences observed between genotypes
 280 and the inconsistency of the changes found associated with the presence of *BMP15* mutations
 281 between breeds led us to investigate whether the components of the inhibin/activin system
 282 could be direct target genes of *BMP15*. As *BMP15* is known to act alone or in interaction
 283 with *GDF9*, and thus activate different SMAD signaling pathways involved in BMP or/and
 284 activin signaling (Mottershead *et al.* 2015), the effects of *BMP15* and *GDF9*, acting alone or
 285 in combination, were compared with those of *BMP4* (a canonical inducer of the BMP
 286 signaling pathway) and *Activin A* (Figure 6). In 48h-culture experiments in serum-free
 287 conditions, *BMP4* and *BMP15* enhanced *INHBA* ($p < 0.001$ and $p < 0.01$, respectively) and *FST*

288 (both $p < 0.001$) expression, but inhibited *TGFBR3* (both $p < 0.001$) expression and had no
289 significant effect on *INHA*, *ACVR1B* and *ACVR2A* mRNA levels. Similarly, Activin A
290 enhanced *FST* ($p < 0.01$) and reduced *TGFBR3* ($p < 0.01$) expression and had no effect on
291 *ACVR1B* expression; in addition, Activin A enhanced *INHA* ($p < 0.01$) expression and, unlike
292 BMPs, had no effect on *INHBA* mRNA levels but inhibited *ACVR2A* ($p < 0.01$) expression.
293 GDF9 alone had no effect on the mRNA levels of all studied genes. However, the
294 combination of BMP15 and GDF9 slightly reduced BMP15 effects on *INHBA* ($p < 0.05$)
295 expression, but enhanced BMP15 effects on *FST* ($p < 0.05$) and *INHA* ($p < 0.05$) expression.

296

297 Discussion

298 To our knowledge, this is the first study dedicated to the regulation by BMP15 of the
299 ovarian expression of the whole inhibin/activin system, i.e. inhibin and activin subunits,
300 activin receptors and antagonists, based on complementary investigations *in vitro* and *in vivo*.
301 We have identified the direct target genes of BMP15 among these components and evaluated
302 the consequence of the presence of *BMP15* loss-of-function mutations on their ovarian
303 expression in two different genetic models of hyperprolific sheep.

304 *In vitro*, the effects of BMP15, BMP4 and Activin A itself were compared on the
305 expression of the different components of the inhibin/activin system. In ovine granulosa cells
306 recovered from small antral follicles, BMP15 (acting alone or in combination with GDF9),
307 BMP4 and Activin A all enhanced *FST* and, in a lesser extent, *INHA* expression, but inhibited
308 the expression of *TGFBR3*. In addition, *INHBA* expression was specifically enhanced by
309 BMP15 and BMP4, whereas *ACVR2A* expression was inhibited by Activin A only. The
310 similar effects of BMP4 and BMP15 can be explained by their common activation of the
311 SMAD1/5/8 signaling pathway (Moore *et al.* 2003), whereas GDF9 and Activin A are known
312 to signal through SMAD2/3 proteins (Mazerbourg *et al.* 2004). From our results, no effect of

313 GDF9 alone was observed, in agreement with the presence of a low activation level of the
314 SMAD2/3 signaling pathway by GDF9 in ovine granulosa cells (Pierre *et al.* 2016). However,
315 GDF9 was found to enhance and decrease the stimulating effects of BMP15 on the expression
316 of *FST* and *INHBA*, respectively. GDF9 and BMP15 are known to form heterodimers (Liao *et*
317 *al.* 2003; McIntosh *et al.* 2008) and exhibit synergistic interactions by activating both SMAD
318 pathways (Mottershead *et al.* 2015). These interactions might be involved in the observed
319 regulations by BMP15 and GDF9, when acting together.

320 *In vivo*, the *FecX^R* mutation, when present at the heterozygous state in the Spanish
321 Rasa Aragonesa breed, was associated with a 1.9-fold increase in ovulation rate, compared to
322 the ovulation rate of non-carrier ewes of the same breed, whereas the recently discovered
323 *FecX^{Gr}* mutation, when present at the homozygous state in the French Grivette breed, led to a
324 2.1-fold increase in ovulation rate, in agreement with previous observations (Lahoz *et al.*
325 2011; Demars *et al.* 2013). From our results, compared to non-carrier ewes, *BMP15* mutant
326 ewes exhibited a higher number of large dominant (preovulatory) follicles during the
327 follicular phase of the estrous cycle, associated with a small decrease in their diameter,
328 suggesting an earlier final differentiation of their granulosa cells, as previously proposed for
329 the Inverdale ewes carrying the *FecX^d* mutation in *BMP15* (McNatty *et al.* 2009) and more
330 strikingly for the Booroola ewes carrying the *FecB^B* mutation in *BMPR1B* (Henderson *et al.*
331 1985). However, there was no clear effect of the *FecX^R* and the *FecX^{Gr}* mutations on
332 *CYP19A1* mRNA levels in the granulosa cells recovered from the two studied follicular size
333 classes, i.e. small (1–3 mm) and large (> or equal to 5 mm) follicles. It is possible that the
334 choice of these follicular classes did not allow detecting any fine difference in the maturation
335 stage of the granulosa cells between genotypes. However, the intermediary class of 3-5 mm
336 follicles was discarded from analysis, due to the high expression of *IGFBP5* and other

337 markers of atresia or apoptosis (Supplementary Figure 2 and Supplementary Table 1) in this
338 size class containing the subordinate follicles already engaged in the atresia process.

339 The presence of loss-of-function *BMP15* mutations *in vivo* had little effect on the
340 expression of the components of the inhibin/activin system and the differences found between
341 genotypes were not consistent with the effects of BMP15 observed on ovine granulosa cells *in*
342 *vitro*. For instance, the expression of *INHA*, *INHBA* and *FST* was not, or only slightly,
343 affected by the presence of *BMP15* mutations *in vivo* in both breeds, despite the existence of a
344 stimulating effect of BMP15 on the expression of these genes *in vitro*. From our *in vitro*
345 results, Activin A was able to enhance the expression of *INHA* and *FST* expression and could
346 such participate in balancing the expression level of these components *in vivo*. However, this
347 hypothesis cannot explain why *INHBA* was unaffected by the presence of *BMP15* mutations
348 *in vivo*.

349 The components of the inhibin/activin system were affected by the presence of a loss-
350 of-function *BMP15* mutation differently in the Rasa Aragonesa and the Grivette breed. For
351 example, in the granulosa cells of the large follicles, *ACVR1B*, *ACVR2A* and *TGFBR3* mRNA
352 levels were lower in the homozygous *FecX^{Gr}*, compared to non-carrier Grivette ewes, but
353 such differences were not observed between the heterozygous *FecX^R* and non-carrier Rasa
354 Aragonesa ewes. Other examples of discrepancies could be cited, concerning Inhibin A
355 concentrations in large follicles and *FST* expression in the granulosa cells of small follicles.
356 These discrepancies might be related to the mutation itself or its homozygous or heterozygous
357 state. The *FecX^R* mutation is known to preclude BMP15 production (Martinez-Royo *et al.*
358 2008) and the *FecX^{Gr}* mutation totally impairs BMP15 signaling in target cells (Demars
359 2013). In both models, a lowering of the biological activity of BMP15 is expected, due to
360 lower amounts of the mature protein (as hypothesized in *R/+* follicles) or lower functional
361 interactions between BMP15 and GDF9 (as hypothesized in *Gr/Gr* follicles), but the

362 molecular mechanisms affecting BMP15 are not yet fully understood. Alternatively, the *in*
363 *vivo* expression level of each component of the inhibin/activin system could depend on the
364 specific equilibrium of the different components existing within each breed. This hypothesis
365 is supported by the observation that the granulosa cells of non-mutant Grivette ewes
366 expressed higher levels of *INHA*, *INHBA*, *ACVR1B* and *ACVR2A*, but lower levels of *FST* and
367 *TGFBR3* mRNA than the granulosa cells of non-mutant Rasa Aragonesa ewes. Consistently,
368 the concentrations of Activin A and Inhibin A proteins were higher in the follicles of Grivette
369 ewes, also illustrating pre-existing differences in the inhibin/activin system between breeds, in
370 the absence of *BMP15* mutation.

371 The complexity of the inhibin/activin system may explain that (1) the effects of
372 BMP15 on granulosa cells *in vitro* are not consistent with the expressional changes observed
373 when a loss-of-function *BMP15* mutation is present *in vivo* (2) the consequences of a *BMP15*
374 mutation differ between breeds. Activin A and its receptors are positive elements of activin
375 signaling, whereas inhibin, follistatin and the inhibin co-receptor called TGFBR3 (or
376 betaglycan) are antagonistic elements. From our *in vitro* results, BMP15 and Activin A itself
377 can acutely regulate the expression of both positive and negative components of the
378 inhibin/activin system. We propose that a re-balancing of the components of this system *in*
379 *vivo* can explain the small effects of *BMP15* loss-of-function mutations and the inconsistency
380 of the changes found associated with the presence of *BMP15* mutations in different genetic
381 backgrounds. Therefore, the inhibin/activin system would not be implicated in the
382 mechanisms leading to multiple ovulations in ewes carrying *BMP15* mutations, in contrast to
383 the recently evidenced AMH system (Pierre *et al.* 2016).

384 In conclusion, the presence of a loss-of-function *BMP15* mutation, such as the *FecX^R*
385 mutation when present at the heterozygous state, or the *FecX^{Gr}* mutation when present at the
386 homozygous state, is associated with a clear increase in ovulation rate leading to a higher

387 prolificacy. In this study, we have challenged a potential role of the ovarian inhibin/activin
388 system in participating in this phenotype. From our *in vitro* results, the genes encoding
389 different components of the inhibin/activin system such as *INHA*, *INHBA*, *FST* and *TGFBR3*
390 are potential targets of BMP15 in ovine granulosa cells. However, *in vivo*, the presence of a
391 loss-of-function *BMP15* mutation in ewes was not found associated with strong changes in the
392 expression of these components in granulosa cells. Moreover, the observed expressional
393 changes were dependent on either the *BMP15* mutation itself or the ovine breed in which the
394 mutation was present. Altogether, these results suggest that the ovarian inhibin/activin system
395 is not a key component of the mechanisms regulating the ovulation rate in *BMP15* mutant
396 ewes.

397

398 **Declaration of interest**

399 The authors declare that there is no conflict of interest that could be perceived as prejudicing
400 the impartiality of the research reported.

401

402 **Funding**

403 This work was supported by grants from France via the “Agence Nationale pour la
404 Recherche” (<http://www.agence-nationale-recherche.fr/>) (ANR-12-BSV1-0034-02,
405 AMHAROC) and EC (FP7/2007–2013), grant 245140, “3SR”, Sustainable Solutions for
406 Small Ruminants (<http://www.3srbreeding.eu/>), WP4 (this publication reflects only the
407 authors’ views and the EC is not liable for any use that may be made of the information
408 contained herein). A. Estienne was supported by a French Fellowship from the Région Centre
409 and INRA.

410

411 **Acknowledgements**

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412 We thank the staff of the slaughterhouse of the CIRE platform at Nouzilly (France) for the
413 recovery of sheep ovaries. We are grateful to the technicians of the INRA experimental unit of
414 Langlade who took care of the Grivette animals in France, and the staff of the experimental
415 animal facilities of CITA de Aragón for taking care and providing the Rasa Aragonesa ewes in
416 Spain.

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Figure legends

Figure 1: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of functional gene markers in the granulosa cells of ewes. Results represent *CYP19A1* (a marker of fully differentiated granulosa cells, upper panels) and *IGFBP5* (a marker of apoptotic granulosa cells, lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and *R/+* (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and *Gr/Gr* (n=5, open bars) Grivette ewes (right panels). All the follicles were recovered on the ovaries of ewes in the follicular phase of a synchronized estrous cycle. *CYP19A1* and *IGFBP5* mRNA accumulation was studied by reverse transcription and quantitative PCR and represented as mRNA relative level, using *RPL19* as an internal reference. In each panel, data were analyzed by one-way ANOVA and different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes.

Figure 2: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding Inhibin A and Activin A subunits in the granulosa cells of ewes. Results represent *INHA* (upper panels) and *INHBA* (lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and *R/+* (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and *Gr/Gr* (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

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Figure 3: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the intra-follicular concentration of Inhibin A and Activin A in sheep ovaries. Results represent the concentrations of Inhibin A (upper panels), Activin A (middle panels) and the Activin A / Inhibin A concentration ratio (lower panels) in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and *R/+* (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and *Gr/Gr* (n=5, open bars) Grivette ewes (right panels). Follicular fluids were recovered on the ovaries of ewes in the follicular phase of a synchronized estrous cycle and hormonal concentrations were measured by ELISA. In each panel, data were analyzed by one-way ANOVA and different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. **: $p < 0.01$, ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

Figure 4: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding activin receptors in the granulosa cells of ewes. Results represent *ACVR1B* (upper panels) and *ACVR2A* (lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and *R/+* (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and *Gr/Gr* (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. *: $p < 0.05$, ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

Figure 5: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding activin/inhibin binding proteins in the granulosa cells of ewes. Results represent *FST* (encoding follistatin, the specific activin-binding protein,

upper panels) and *TGFBR3* (encoding betaglycan, the co-receptor of inhibin, lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from *+/+* (n=6, black bars) and *R/+* (n=6, open bars) Rasa Aragonesa ewes (left panels), and *+/+* (n=5, black bars) and *Gr/Gr* (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. *: $p < 0.05$, **: $p < 0.01$ vs. the corresponding follicle class in *+/+* Rasa Aragonesa ewes.

Figure 6: Effect of BMP4, BMP15, GDF9 and Activin A on the expression of genes encoding different members of the inhibin/activin system in ovine granulosa cells. Sheep granulosa cells were cultured in 96-well plates for 48 hours with or without 50 ng/ml of the different factors. Each treatment was tested in 12 replicate wells, then cells were pooled according to treatment at the end of culture for mRNA preparation. Messenger RNA accumulation of *INHA*, *INHBA*, *ACVR1B*, *ACVR2A*, *FST* and *TGFBR3* was studied by reverse transcription and quantitative PCR relatively to *RPL19* as internal reference. The results were expressed in percentage of expression relatively to that of cells cultured without ligand (Ctrl, fixed at 100%). Data represent the results of 15 independent experiments performed for each treatment. Comparisons of means between different treatments were made by repeated measures one-way ANOVA with the Greenhouse-Geisser correction of the degrees of freedom, followed by Fisher's LSD test for multiple comparisons. (*): $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, vs. Ctrl, or between BMP15 and BMP15+GDF9 conditions.

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Supplementary Figure 1: Parallelism of dilution curves of ovine plasma and follicular fluids with the standard curves in the Active Inhibin A ELISA kit (Panel A) and in the Human/Mouse/Rat Activin A Quantikine ELISA kit (Panel B). The panels depict the results of the serial dilutions of ovine follicular fluid samples (FF1, FF2 and FF3) and plasma from 3 ewes (E 950428, E 982067 and E 020720) and the corresponding standard curves (SC) of the Inhibin A and Activin A ELISA. Dilutions of the standard points and the ovine samples were all made in steer plasma. The absorbance (optical density) measured at 450 nm at the end of the test is directly proportional to the concentration of Inhibin A (Panel A) or Activin A (Panel B) in the samples.

Supplementary Figure 2: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the mRNA expression of 3 gene markers of follicular atresia in the granulosa cells of ewes. All the follicles were recovered on the ovaries of ewes in the follicular phase of a synchronized estrous cycle. *IGFBP5*, *GADD45A* and *CLU* mRNA accumulation was studied by reverse transcription and quantitative PCR and represented as mRNA relative level, using *RPL19* as an internal reference. The cycle thresholds (Ct) of *RPL19* are shown in the lower panels. The specific primer sequences used for amplification are listed in Supplementary Table 1. In each panel, data were analyzed by one-way ANOVA and different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes.

Table 1: Quantitative PCR primers sequences and their efficiency

Gene	Accession number	Forward sequence 5'→3'	Reverse sequence 5'→3'	Efficiency
<i>CYP19A1</i>	NM_001123000.1	TCATCCTGGTCACCCTTCTG	CCAGACGAGACCAGCGACCG	1.95
<i>IGFBP5</i>	NM_001129733.1	CTACAAGAGAAAAGCAGTG	CAGCTTCATCCCATACTT	1.99
<i>INHA</i>	NM_001308579.1	GACCAAGATGTCTCCCAG	CAGTATGGAACCACAGCT	2.00
<i>INHBA</i>	NM_001009458.1	GAAGAGGAAAGAAGAGGAA	GTAGTGGTTGATGACTGT	2.08
<i>ACVR1B</i>	XM_012174234.2	TTGTGTACTGGGAGATTG	ACTAGGTCATAATATGGCAG	1.96
<i>ACVR2A</i>	NM_001009293.1	GATTAGTCCTGTGGGAAC	GTCTTCAAGAGAGGGATG	2.05
<i>FST</i>	NM_001257093.1	TGGACCAGACTAATAATG	TATCTTCACAGGACTTTG	1.87
<i>TGFβR3</i>	XM_012176015.2	CTCTTTCTCCAGTGTGAG	GATCATTGAGGCATCCAG	1.98
<i>RPL19</i>	XM_004012837	AATGCCAATGCCAACTC	CCCTTTCGTACCTATAACC	1.95

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- 1 Table 2: Follicular populations in the ovaries of Rasa Aragonesa and Grivette ewes with
2 different genotypes

Breed Genotype (N° of ewes)	Rasa Aragonesa		Grivette	
	+/+ (n=6)	R/+ (n=6)	+/+ (n=5)	Gr/Gr (n=5)
N° of small follicles (1-3 mm)	43.0 ± 7.4	42.8 ± 6.5	48.2 ± 3.7	56.6 ± 6.8
N° of large follicles (> or equal to 5 mm)	2.3 ± 0.3	3.7 ± 0.3*	2.2 ± 0.2	3.8 ± 0.9
Mean diameter of the large follicles (mm)	7.25 ± 0.33	5.88 ± 0.11**	7.06 ± 0.28	5.36 ± 0.09***

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- 4 *: p<0.05, **: p<0.01; ***: p<0.001, vs. +/+ ewes of the same breed.

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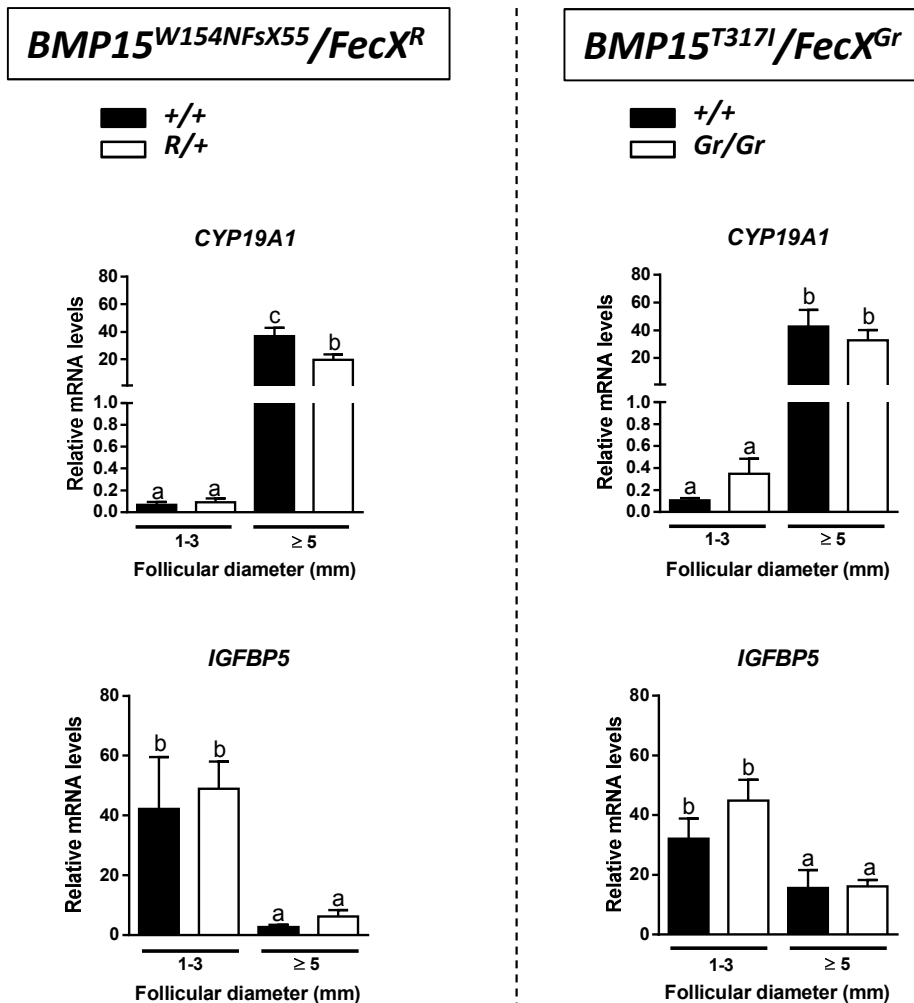


Figure 1: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of functional gene markers in the granulosa cells of ewes. Results represent *CYP19A1* (a marker of fully differentiated granulosa cells, upper panels) and *IGFBP5* (a marker of apoptotic granulosa cells, lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and R/+ (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and Gr/Gr (n=5, open bars) Grivette ewes (right panels). All the follicles were recovered on the ovaries of ewes in the follicular phase of a synchronized estrous cycle. *CYP19A1* and *IGFBP5* mRNA accumulation was studied by reverse transcription and quantitative PCR and represented as mRNA relative level, using *RPL19* as an internal reference. In each panel, data were analyzed by one-way ANOVA and different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes.

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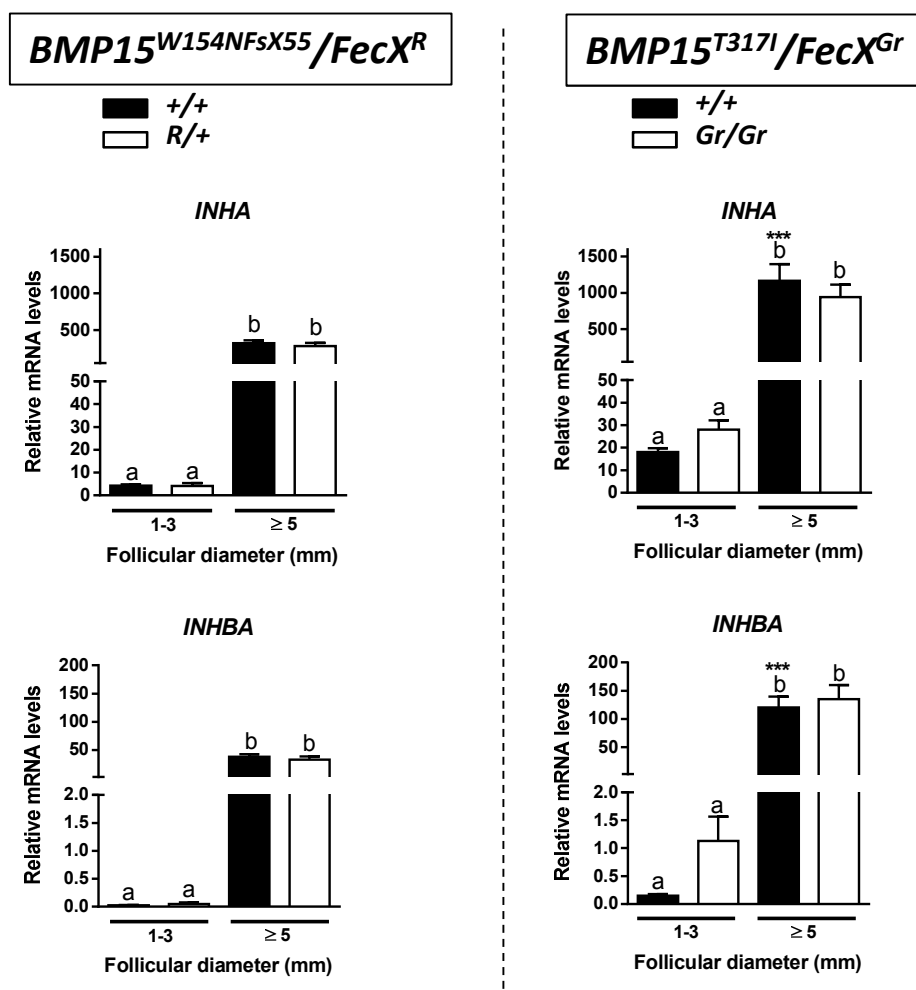


Figure 2: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding Inhibin A and Activin A subunits in the granulosa cells of ewes. Results represent *INHA* (upper panels) and *INHBA* (lower panels) expression in small growing (1-3 mm) and large dominant (≥ 5 mm) follicles from +/+ (n=6, black bars) and R/+ (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and Gr/Gr (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

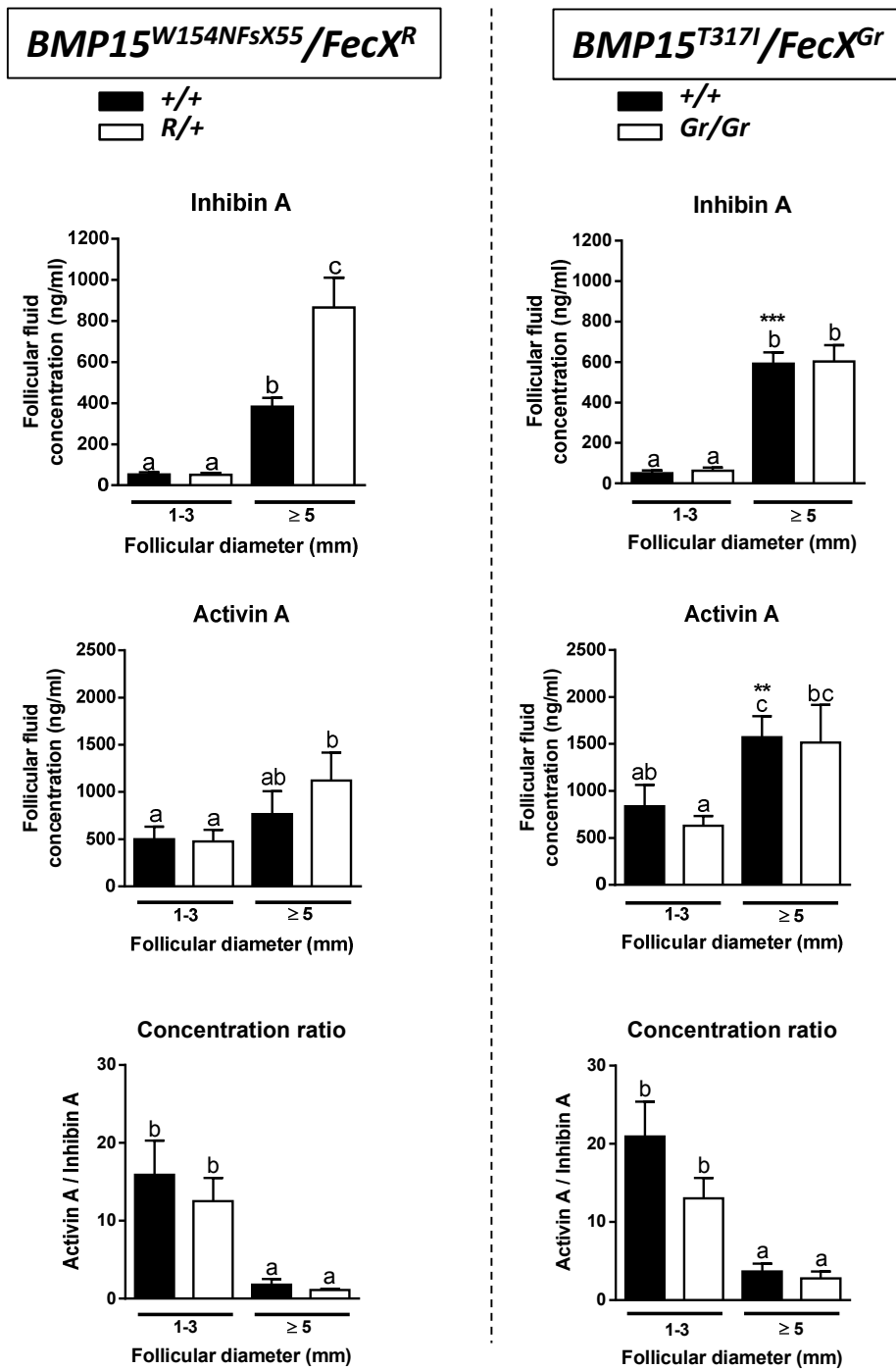


Figure 3: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the intra-follicular concentration of Inhibin A and Activin A in sheep ovaries. Results represent the concentrations of Inhibin A (upper panels), Activin A (middle panels) and the Activin A / Inhibin A concentration ratio (lower panels) in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and R/+ (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and Gr/Gr (n=5, open bars) Grivette ewes (right panels). Follicular fluids were recovered on the ovaries of ewes in the follicular phase of a synchronized estrous cycle and hormonal concentrations were measured by ELISA. In each panel, data were analyzed by one-way ANOVA and different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. **: $p < 0.01$, ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

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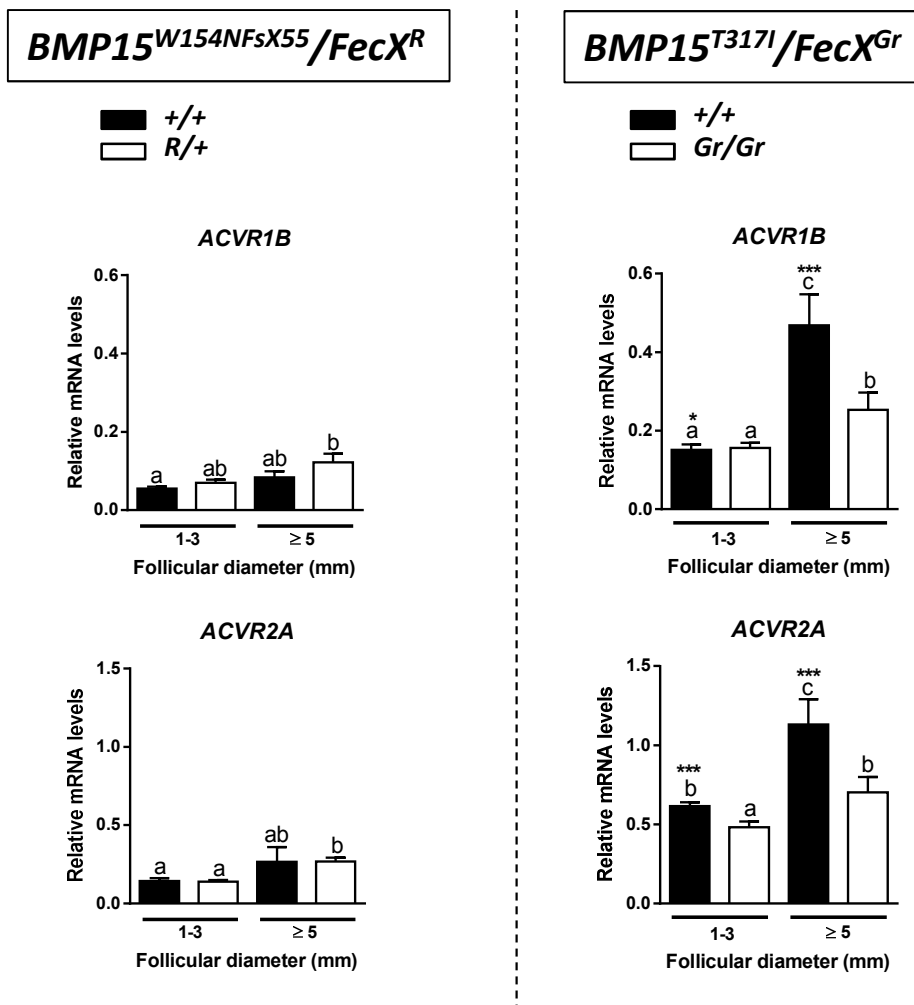


Figure 4: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding activin receptors in the granulosa cells of ewes. Results represent *ACVR1B* (upper panels) and *ACVR2A* (lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and R/+ (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and Gr/Gr (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. *: $p < 0.05$, ***: $p < 0.001$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

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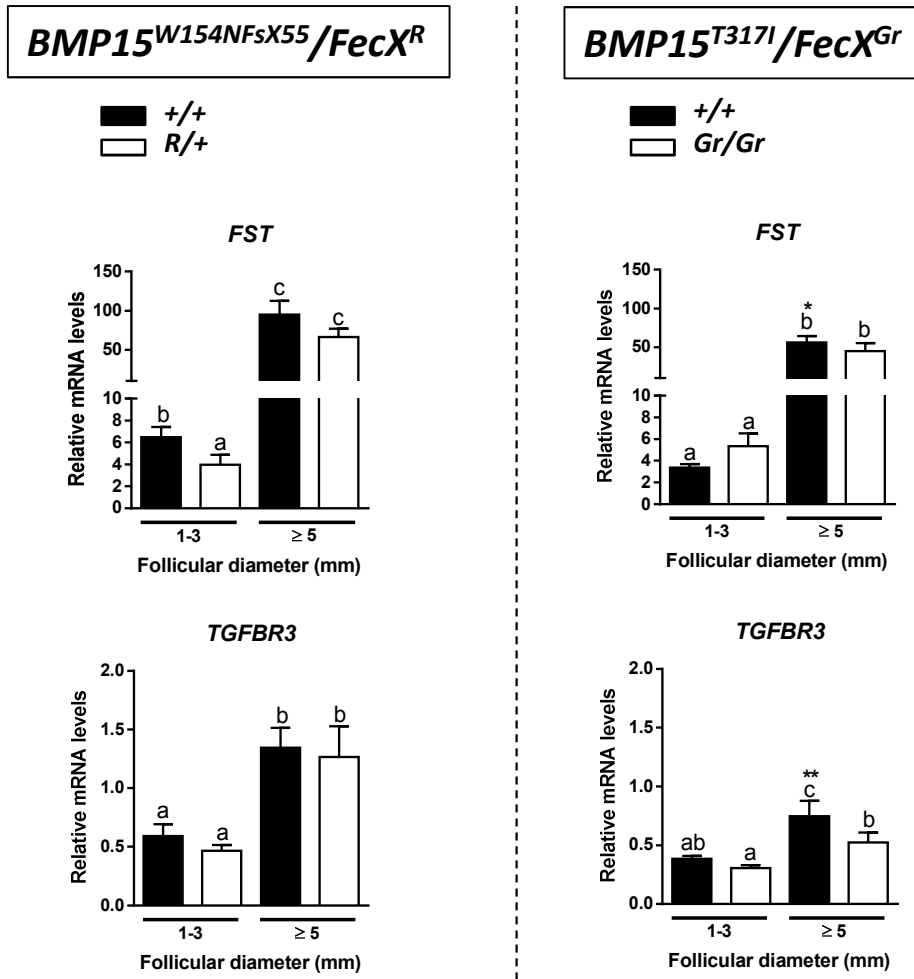


Figure 5: Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding activin/inhibin binding proteins in the granulosa cells of ewes. Results represent *FST* (encoding follistatin, the specific activin-binding protein, upper panels) and *TGFBR3* (encoding betaglycan, the co-receptor of inhibin, lower panels) expression in small growing (1-3 mm) and large dominant (> or equal to 5 mm) follicles from +/+ (n=6, black bars) and R/+ (n=6, open bars) Rasa Aragonesa ewes (left panels), and +/+ (n=5, black bars) and Gr/Gr (n=5, open bars) Grivette ewes (right panels), analyzed as described in Figure 1. In each panel, different letters indicate significant differences ($p < 0.05$) between follicular classes and genotypes. *: $p < 0.05$, **: $p < 0.01$ vs. the corresponding follicle class in +/+ Rasa Aragonesa ewes.

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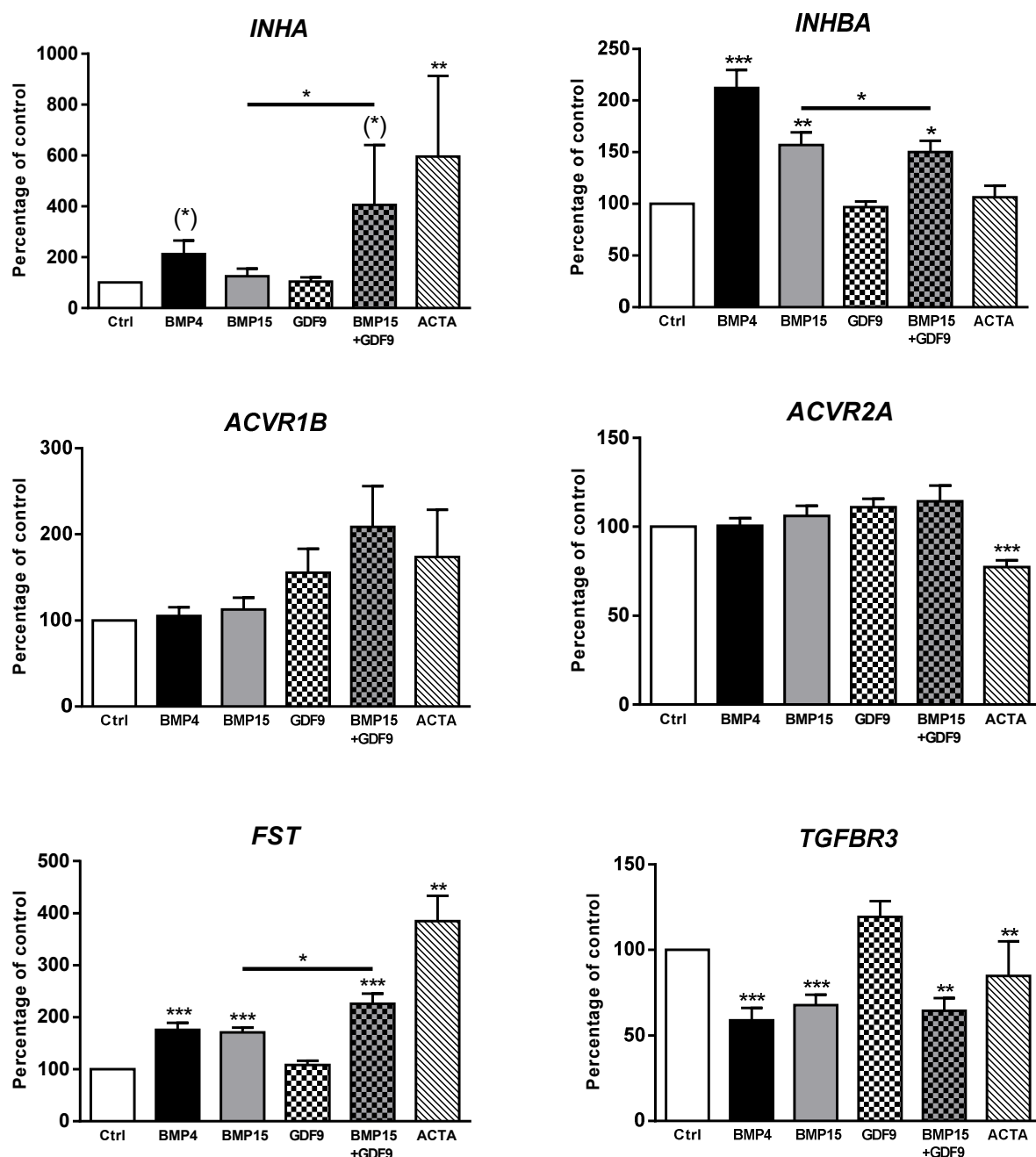


Figure 6: Effect of BMP4, BMP15, GDF9 and Activin A on the expression of genes encoding different members of the inhibin/activin system in ovine granulosa cells. Sheep granulosa cells were cultured in 96-well plates for 48 hours with or without 50 ng/ml of the different factors. Each treatment was tested in 12 replicate wells, then cells were pooled according to treatment at the end of culture for mRNA preparation. Messenger RNA accumulation of *INHA*, *INHBA*, *ACVR1B*, *ACVR2A*, *FST* and *TGFBR3* was studied by reverse transcription and quantitative PCR relatively to *RPL19* as internal reference. The results were expressed in percentage of expression relatively to that of cells cultured without ligand (Ctrl, fixed at 100%). Data represent the results of 15 independent experiments performed for each treatment. Comparisons of means between different treatments were made by repeated measures one-way ANOVA with the Greenhouse-Geisser correction of the degrees of freedom, followed by Fisher's LSD test for multiple comparisons. (*): $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, vs. Ctrl, or between BMP15 and BMP15+GDF9 conditions.

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