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

Anthony Estienne, Belén Lahoz, Peggy Jarrier, Loys Bodin, José Folch, José-Luis Alabart, Stéphane Fabre and Danielle Monniaux

1UMR85 PRC, INRA, CNRS, IFCE, Université de Tours, Nouzilly, France, 2Unidad de Producción y Sanidad Animal, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Zaragoza, España, and 3GenPhySE, Université de Toulouse, INRA, INPT, ENV, Castanet Tolosan, France

Correspondence should be addressed to D Monniaux; Email: Danielle.Monniaux@inra.fr

Abstract

Polymorphisms in the gene encoding bone morphogenetic protein 15 (BMP15) have been associated with multiple ovulations in sheep. As BMP15 regulates inhibin expression in rodents, we assumed that the ovarian inhibin/activin system could mediate part of the effect of BMP15 mutations in the regulation of ovulation rate in sheep. To answer this question, we have studied the effects of two natural loss-of-function mutations of BMP15 on the expression of components of this system. The FecX8 and the FecX0 mutations, when present respectively in Rasa Aragonesa ewes at the heterozygous state and in Grivette ewes at the homozygous state, were associated with a twofold increase in ovulation rate. There were only small differences between mutant and wild-type ewes for mRNA expression of INHA, INHBA, ACVR1B, ACVR2A, FST or TGFBR3 in granulosa cells and inhibin A or activin A concentrations in follicular fluid. Moreover, the effects of mutations differed between breeds. In cultures of granulosa cells from wild-type ewes, BMP15, acting alone or in synergy with GDF9, stimulated INHBA and INHA follicular fluid. Moreover, the effects of mutations differed between breeds. In cultures of granulosa cells from wild-type ewes, BMP15, acting alone or in synergy with GDF9, stimulated INHBA and INHA and FST expression, but inhibited the expression of TGFBR3. Activin A did not affect INHBA expression, but inhibited the expression of ACVR2A also. The complexity of the inhibin/activin system, including positive and antagonistic elements, and the differential regulation of these elements by BMP15 and activin can explain that the effects of BMP15 mutations differ when present in different genetic backgrounds. In conclusion, the ovarian inhibin/activin system is unlikely to participate in the increase of ovulation rate associated with BMP15 mutations in sheep.


Introduction

Increasing evidence support the involvement of cytokines belonging to the transforming growth factor beta (TGFβ) family in the control of ovulation number at each sexual cycle (ovulation rate) in adult females. In particular, the bone morphogenetic proteins BMP15 (bone morphogenetic protein 15) and GDF9 (growth differentiation factor 9), known to be specifically expressed in the oocyte (McGrath et al. 1995, Dube et al. 1998), could play a key role in this process. In sheep, heterozygous carriers of various missense mutations affecting BMP15 or GDF9 or carriers of a partial loss-of-function mutation in their receptor BMPR1B, exhibit systematically increased ovulation rates and litter sizes (reviews: Montgomery et al. 2001, Galloway et al. 2002, McNatty et al. 2003, Fabre et al. 2006, Persani et al. 2014). Polymorphisms in GDF9, BMPR1B and SMAD3 (encoding a BMP15/GDF9 signaling factor) have also been shown to occur among human cohorts with a propensity for dizygotic twins (Palmer et al. 2006, Hoekstra et al. 2008, Luong et al. 2011, Mbarek et al. 2016), and the presence of non-synonymous substitutions in BMP15 or GDF9 genes has been recently associated with twinning in callitrichine primates (Harris et al. 2014). From a phylogenetic analysis comparing mono- and poly-ovulating mammalian species, high variations have been observed in specific areas of BMP15 and GDF9, which are able to modify the biological activity of the proteins in poly-ovulating mammals (Monestier et al. 2014).

The mechanisms by which BMP15 and GDF9 control the ovulation rate are not fully understood. BMP15, acting alone or in synergy with GDF9, can modulate follicle growth and maturation by controlling granulosa cell proliferation and their responsiveness to FSH (Otsuka et al. 2000, 2001, Moore et al. 2003, McNatty et al. 2005b, Moore & Shimasaki 2005). Recently, the anti-Müllerian hormone (AMH), another member of the TGFβ family known to importantly modulate FSH sensitivity (Durlinger et al. 2001, Visser & Themmen 2005, 2014, Knight & Glista 2006), has also been proposed to participate in this mechanism.
Indeed, BMP15 enhances the expression of AMH and its specific receptor AMHR2 in the granulosa cells of sheep antral follicles, and hyperprolific ewes carrying a loss-of-function mutation in BMP15 or its receptor BMPR1B show respectively impaired expression of AMHR2 or AMH (Estienne et al. 2015, Pierre et al. 2016). It is suggested that low BMP15 and low AMH signaling in follicles of these mutant ewes contribute to sensitizing granulosa cells to FSH, thereby promoting the selection and maintenance of follicles during the follicular phase, and thus enhancing the ovulation rate.

Inhibins and activins, other members of the TGFβ family, could also be involved in the mechanisms leading to multiple ovulations. Inhibins and activins are produced by the granulosa cells of growing follicles and known to act in an opposite way on their target cells. Activins are importantly implicated in follicular growth, acting in an intra-ovarian way by promoting granulosa cell proliferation, enhancing FSHR (follicle-stimulating hormone receptor) and CYP19A1 (cytochrome P450 family 19 subfamily A member 1, also known as aromatase) expression, and inhibiting follicle atresia and luteinization, whereas inhibins oppose the activin signaling, thus modulating these processes (Knight et al. 2012). Inhibins are secreted by the largest follicles during terminal follicular development and are important inhibitors of FSH secretion by pituitary gonadotrophs. In various animal species, immunization against inhibin induces multiple ovulations and an increase in pituitary secretion of FSH appears to be the main mechanism by which the growth of additional follicles is stimulated (Henderson et al. 1984, Forage et al. 1987, Hillard et al. 1995, Drummond et al. 2004, Yan et al. 2015). However, immunization against inhibin is not always accompanied by the expected rise in plasma FSH, and intra-ovarian effects of neutralizing endogenous inhibin bioactivity might also be involved in the stimulation of follicular development (Tannetta et al. 1998). Interestingly, the specific intra-ovarian glycosylation of the inhibin alpha and beta A subunits is associated with multiple ovulations and high prolificacy in ewes carrying the FecL5 mutation, which induces an ectopic expression of the B4GALNT2 (Beta-1,4-N-acetylgalactosaminyl transferase 2) gene within the ovary (Drouilhet et al. 2013).

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

Materials and methods

Animals

For in vivo expression studies, granulosa cells were recovered from hyperprolific ewes carrying mutations in the BMP15 gene and their corresponding non-carrier ewes of the same breed. The BMP15W154NFsX55/FecXR mutation is a 17 bp deletion creating a premature stop codon in the BMP15 pro-region; it causes hyperprolificacy in the Spanish Rasa Aragonesa breed when the mutation is present in ewes at the heterozygous state and sterility when present at the homozygous state (Martinez-Royo et al. 2008). The BMP15T317I/FecXGr mutation is a substitution of a threonine for an isoleucine, which affects the hydrophobicity of the protein and impairs its signaling activity; it causes hyperprolificacy in the French Grivette breed when the mutation is present at the heterozygous state and even more at the homozygous state (Demars et al. 2013). In this study, 6 Rasa Aragonesa ewes heterozygous carriers of the FecXGr mutation (R+/; ovulation rate = 1.89 ± 0.16), 6 Rasa Aragonesa non-carrier ewes (+/+; ovulation rate = 1.00), 5 Grivette ewes homozygous carriers of the FecXGr mutation (Gr/Gr; ovulation rate = 4.88 ± 0.55) and 5 Grivette non-carrier ewes (+/+; ovulation rate = 2.29 ± 0.20) were used after genotyping.

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

Collection and culture of granulosa cells

Collected ovaries were finely dissected to isolate all the antral follicles larger than 1 mm in diameter. For each animal, the follicles were then classified according to their size and genotype. For the evaluation of in vivo gene expression in
Rasa Aragonesa and Grivette ewes, two follicular classes were defined, small (1–3 mm) and large (larger than or equal to 5 mm) follicles, corresponding to follicle growth and dominance stages respectively. Follicular fluid and granulosa cells were recovered from small and large follicles as described (Estienne et al. 2015). For small follicles, follicular fluid and granulosa cells were pooled within animal. The volumes of follicular fluid recovered by follicular puncture from each animal were at least 50 µL for individual large follicles and 5 µL for pooled small follicles. Follicular fluids and granulosa cells were stored at −80°C for further analysis.

For culture experiments, ovaries were recovered from ewes of various breeds wildtype at the two BMP15 loci after slaughtering in a local slaughterhouse at Nouzilly (France). Granulosa cells from 1 to 3 mm follicles were cultured in serum-free conditions in McCoy’s 5a medium (Sigma) containing insulin (100 ng/mL) according to a previously described method (Campbell 1996). Cells were seeded at 10^5 viable cells/well in 96-well plates with and without recombinant human BMP4, human BMP15, mouse GDF9 or human/mouse/rat Activin A (50 ng/mL each; R&D System Europe), each treatment being tested in 12 replicate wells. After 48 h of culture, cells were recovered using Accutase (Sigma), and then pooled according to treatment, centrifuged and cell pellets were stored at −80°C for further RNA extraction. In total, 15 independent culture experiments were performed.

**RNA extraction and quantitative real-time PCR**

Samples of granulosa cell recovered in vivo and after culture were analyzed for mRNA content of genes encoding components of the inhibin/activin system (INHA, INHBA, ACVR1B, ACVR2A, FST and TGFBR3). For in vivo gene expression maturation and health was assessed using classic markers of differentiation (CYP19A1) and atresia (IGFBP5) in ovine granulosa cells (Besnard et al. 1996, Logan et al. 2002). Total RNA was extracted with a Nucleospin RNA II kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's protocol.

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

**Inhibin and activin assays**

Inhibin A concentration in follicular fluid was measured using the Active Inhibin A ELISA kit (Beckman Coulter France) according to the manufacturer’s protocol. The use of the Active Inhibin A ELISA kit in sheep has been validated by studying serial dilutions of different ovine plasma and follicular fluid samples in steer plasma. Results showed that the dilution curves were linear and parallel to the standard curve (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). In the present study, inhibin A concentrations were determined in 30 µL aliquots of follicular fluid diluted at 1/100 and 1/1000 for small and large follicles respectively. In our working conditions, the limit of detection of the assay was found to be 0.5 pg/well and the intra- and inter-assays coefficients of variation were lower than 5%.

Activin A concentration in follicular fluid was measured using the Human/Mouse/Rat Activin A Quantikine ELISA kit (R&D Systems Europe) according to the manufacturer’s protocol. The use of the Human/Mouse/Rat Activin A Quantikine ELISA kit in sheep has been validated by studying serial dilutions of different ovine plasma and follicular fluid samples in steer plasma. Results showed that the dilution curves were linear and parallel to the standard curve (Supplementary Fig. 1B). In the present study, Activin A concentrations were determined in 100 µL aliquots of follicular fluid diluted at 1/1000 and 1/10000 for small and large follicles respectively. In our working conditions, the limit of detection of the assay was found to be 1.5 pg/well, and the intra- and inter-assays coefficients of variation were lower than 5% and 7% respectively.

**Statistical analysis**

All data are presented as mean±s.e.m. Data were analyzed using the GraphPad Prism 6 Software (GraphPad Software). In the wild-type (+/+ ewes) comparisons of follicle numbers and sizes between the Rasa Aragonesa and the Grivette breeds were made using Mann–Whitney test, and comparisons of gene expression and hormonal concentrations in follicular fluid between breeds and follicular size classes were made using

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**Table 1** Quantitative PCR primers sequences and their efficiency.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward sequence 5′→3′</th>
<th>Reverse sequence 5′→3′</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19A1</td>
<td>NM_001123000.1</td>
<td>TCATCCGTGTCACCTCTCTG</td>
<td>CAGACGAGACCGCGAGCGG</td>
<td>1.95</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>NM_001129733.1</td>
<td>CTACAAAGAGAACAGCTT</td>
<td>CAGGTCGCCAGCAGACGT</td>
<td>1.99</td>
</tr>
<tr>
<td>INHBA</td>
<td>NM_001308579.1</td>
<td>GACCAAGATGCTCCCAAGCC</td>
<td>CGATGATGGTGGAT</td>
<td>2.00</td>
</tr>
<tr>
<td>ACVR1B</td>
<td>XM_012172434.2</td>
<td>TTGTTCACTGGGAGATTC</td>
<td>ACTACGTCATAATGCGAC</td>
<td>2.08</td>
</tr>
<tr>
<td>ACVR2A</td>
<td>NM_001099239.1</td>
<td>GATTAGTCTGGGAGAACAGCC</td>
<td>GTACCTGACAGATTC</td>
<td>2.05</td>
</tr>
<tr>
<td>FST</td>
<td>NM_001257093.1</td>
<td>TGGACCAAGCTATATG</td>
<td>TTCTCAGAGAGGGAT</td>
<td>1.87</td>
</tr>
<tr>
<td>TGFBR3</td>
<td>XM_012176015.2</td>
<td>CTCCTTCAGGTGGTGGAG</td>
<td>GATCGGAGGATCGGC</td>
<td>1.98</td>
</tr>
<tr>
<td>RPL19</td>
<td>XM_004012837</td>
<td>AATGCAAATGCAAATCT</td>
<td>CCGCTTGCACCTATACC</td>
<td>1.95</td>
</tr>
</tbody>
</table>
two-way ANOVA. Within each ovine breed, gene expression and hormonal concentrations were compared between follicular classes and genotypes using two-way ANOVA. The ANOVA analyses were followed by Fisher’s protected least significant difference (LSD) test for multiple comparisons. For granulosa cell culture experiments, data were analyzed using repeated measures one-way ANOVA with the Greenhouse–Geisser correction of the degrees of freedom. For all analyses, a probability of at least 0.05 was required for significance.

**Results**

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

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

In +/+ ewes, there was no difference between the Rasa Aragonesa and Grivette breeds for CYP19A1 (a marker of follicle maturation) and IGFBP5 (a marker of follicle atresia) expression in granulosa cells. As expected, in both breeds and all genotypes, CYP19A1 mRNA levels were strongly increased in the granulosa cells of the large follicles, compared to those in the small ones (P<0.001 for all comparisons, Fig. 1). This increase in granulosa cell differentiation was associated with a lower expression of the atresia marker IGFBP5 in the large follicles (Rasa Aragonesa breed: P<0.001 for both +/+ and R/+ follicles; Grivette breed: P<0.05 for +/+ follicles and P<0.01 for Gr/Gr follicles). There was no difference between genotypes, except a 1.9-fold lower CYP19A1 expression in R/+ than that in +/+ large follicles, in the Rasa Aragonesa breed (P<0.05, Fig. 1).

![Figure 1](image-url) **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 (larger than 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 two-way ANOVA, and different letters indicate significant differences (P<0.05) between follicular classes and genotypes.

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**Table 2** Follicular populations in the ovaries of Rasa Aragonesa and Grivette ewes with different genotypes.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Rasa Aragonesa</th>
<th>Grivette</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+ (n=6)</td>
<td>R/+ (n=6)</td>
</tr>
<tr>
<td>No. of small follicles (1–3 mm)</td>
<td>43.0±7.4</td>
<td>42.8±6.5</td>
</tr>
<tr>
<td>No. of large follicles (larger than or equal to 5 mm)</td>
<td>2.3±0.3</td>
<td>3.7±0.3*</td>
</tr>
<tr>
<td>Mean diameter of the large follicles (mm)</td>
<td>7.25±0.33</td>
<td>5.88±0.11**</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001 vs +/+ ewes of the same breed.
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to the small ones (P<0.001 for both genes and all comparisons, Fig. 2). Within each breed, there was no difference between genotypes for INHA and INHBA mRNA levels.

The intra-follicular concentrations of inhibin A and activin A were both higher (P<0.001 and P<0.01 respectively) in +/+ large follicles of the Grivette, compared to those in the Rasa Aragonesa breed, but the activin A/INHA concentration ratio was similar between breeds. In both breeds and all genotypes, the intra-follicular concentrations of inhibin A were higher in the large follicles, compared to those in the small ones (P<0.001 for all comparisons) and, except for the Rasa Aragonesa +/+ ewes, the intra-follicular concentrations of activin A were also significantly higher in the large follicles (P<0.05 for all comparisons, Fig. 3). In both breeds and all genotypes, the activin A/INHA concentration ratio was lower in the large follicles, compared to that in the small ones (P<0.001 for all comparisons). No significant differences were found between genotypes, except a 2.3-fold higher

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 (larger than 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 Fig. 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 (larger than 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 two-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.

Concentration ratio

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

The mRNA levels of ACVR1B and ACVR2A were higher in +/+ granulosa cells from large follicles (P<0.001 for both genes) and small follicles (P<0.05 and P<0.001
for ACVR1B and ACVR2A respectively) of the Grivette, compared to the Rasa Aragonesa breed. In the Grivette breed, ACVR1B and ACVR2A mRNA levels were higher in the granulosa cells of the large follicles, compared to the small ones for both genotypes (ACVR1B and ACVR2A: P < 0.001 for +/+ follicles and P < 0.05 for Gr/Gr follicles, Fig. 4), but there was no significant difference between follicular sizes in the Rasa Aragonesa breed, except higher ACVR2A mRNA levels in the large R/+ follicles, compared to the small ones of the same genotype (P < 0.01). In the Grivette breed, ACVR1B mRNA levels were 1.9-fold lower (P < 0.01) and ACVR2A mRNA levels were 1.6-fold lower (P < 0.01) in Gr/Gr, compared to +/+ large follicles (Fig. 4), but no difference between genotypes was observed in the Rasa Aragonesa breed.

The mRNA levels of FST and TGFBR3 were lower (P < 0.05 and P < 0.01 respectively) in +/+ granulosa cells from large follicles of the Grivette, compared to the Rasa Aragonesa breed. In both breeds and all genotypes, FST and TGFBR3 mRNA levels were increased in the granulosa cells of the large follicles, compared to the small ones (FST: P < 0.001 for both breeds and all genotypes, TGFBR3: P < 0.001 for both genotypes in Rasa Aragonesa breed, P < 0.001 for +/+ and P < 0.01 for Gr/Gr genotypes in Grivette breed, Fig. 5). FST mRNA levels were 1.6-fold lower (P < 0.05) in R/+ compared to +/+ small follicles in the Rasa Aragonesa breed (Fig. 5), but no difference in FST expression was observed between genotypes in the Grivette breed. TGFBR3 mRNA levels were 1.4-fold lower (P < 0.05) in Gr/Gr, compared to +/+ large follicles in the Grivette breed (Fig. 5), but no difference between genotypes was observed in the Rasa Aragonesa breed.

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

The small (<3-fold changes), or non-existent differences observed between genotypes and the inconsistency
of the changes found associated with the presence of BMP15 mutations between breeds led us to investigate whether the components of the inhibin/activin system could be direct target genes of BMP15. As BMP15 is known to act alone or in interaction with GDF9, and thus activate different SMAD signaling pathways involved in BMP or/and activin signaling (Mottershead et al. 2015), the effects of BMP15 and GDF9, acting alone or in combination, were compared with those of BMP4 (a canonical inducer of the BMP signaling pathway) and Activin A (Fig. 6). In 48h-culture experiments in serum-free conditions, BMP4 and BMP15 enhanced INHBA (P<0.001 and P<0.01 respectively) and FST (both P<0.001) expression, but inhibited TGFBR3 (both P<0.001) expression and had no significant effect on INHA, ACVR1B and ACVR2A mRNA levels. Similarly, Activin A enhanced FST (P<0.01) expression but reduced TGFBR3 (P<0.01) expression and had no effect on ACVR1B expression; in addition, Activin A enhanced INHA (P<0.01) expression and, unlike BMPs, had no effect on INHA mRNA levels but inhibited ACVR2A (P<0.001) expression. GDF9 alone had no effect on the mRNA levels of all studied genes. However, the combination of BMP15 and GDF9 slightly reduced BMP15 effects on INHBA (P<0.05) expression, but enhanced BMP15 effects on FST (P<0.05) and INHA (P<0.05) expression.

**Discussion**

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

In vitro, the effects of BMP15, BMP4 and activin A itself were compared on the expression of the different components of the inhibin/activin system. In ovine granulosa cells recovered from small antral follicles, BMP15 (acting alone or in combination with GDF9), BMP4 and activin A all enhanced FST and, in a lesser extent, INHA expression, but inhibited the expression of TGFBR3. In addition, INHBA expression was specifically enhanced by BMP15 and BMP4,
whereas ACVR2A expression was inhibited by activin A only. The similar effects of BMP4 and BMP15 can be explained by their common activation of the SMAD1/5/8 signaling pathway (Moore et al. 2003), whereas GDF9 and activin A are known to signal through SMAD2/3 proteins (Mazerbourg et al. 2004). From our results, no effect of GDF9 alone was observed, in agreement with the presence of a low activation level of the SMAD2/3 signaling pathway by GDF9 in ovine granulosa cells (Pierre et al. 2016). However, GDF9 was found to enhance and decrease the stimulating effects of BMP15 on the expression of FST and INHBA respectively. GDF9 and BMP15 are known to form heterodimers (Liao et al. 2003, McIntosh et al. 2008) and exhibit synergistic interactions by activating both SMAD pathways (Mottershead et al. 2015). These interactions might be involved in the observed regulations by BMP15 and GDF9, when acting together.

In vivo, the FecX<sup>R</sup> mutation, when present at the heterozygous state in the Spanish Rasa Aragonesa breed, was associated with a 1.9-fold increase in ovulation rate, compared to the ovulation rate of non-carrier ewes of the same breed, whereas the recently discovered FecX<sup>C</sup> mutation, when present at the homozygous state in the French Grivette breed, led to a 2.1-fold increase in ovulation rate, in agreement with previous observations (Lahoz et al. 2011, Demars et al. 2013). From our results, compared to non-carrier ewes, BMP15 mutant ewes exhibited a higher number of large dominant (preovulatory) follicles during the follicular phase of the estrous cycle, associated with a small decrease in their diameter, suggesting an earlier final differentiation of their granulosa cells, as previously proposed for the Inverdale ewes carrying the FecX<sup>R</sup> mutation in BMP15 (McNatty et al. 2009) and more strikingly for the Boorooala ewes carrying the FecB<sup>B</sup> mutation in BMP1R1B (Henderson et al. 1985). However, there was no clear effect of the FecX<sup>R</sup> and the FecX<sup>C</sup> mutations on CYP19A1 mRNA levels in the granulosa cells recovered from the two studied follicular size classes, i.e. small (1–3 mm) and large (less than or equal to 5 mm) follicles. It is possible that the choice of these follicular classes did not allow detecting any fine difference in the maturation stage of the granulosa cells between genotypes. However, the intermediary class of 3–5 mm follicles was discarded from analysis, due to the high expression of IGFBP5 and other markers of atresia or apoptosis (Supplementary Fig. 2 and Supplementary Table 1) in this size class containing the subordinate follicles already engaged in the atresia process.

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

The components of the inhibin/activin system were affected by the presence of a loss-of-function BMP15 mutation differently in the Rasa Aragonesa and the Grivette breed. For example, in the granulosa cells of the large follicles, ACVR1B, ACVR2A and TGFBR3 mRNA levels were lower in the homozygous FecX<sup>C</sup>, compared to non-carrier Grivette ewes, but such differences were not observed between the heterozygous FecX<sup>C</sup> and non-carrier Rasa Aragonesa ewes. Other examples of discrepancies could be cited, concerning inhibin A concentrations in large follicles and FST expression in the granulosa cells of small follicles. These discrepancies might be related to the mutation itself or its homozygous or heterozygous state. The FecX<sup>R</sup> mutation is known to preclude BMP15 production (Martinez-Royo et al. 2008), and the FecX<sup>C</sup> mutation totally impairs BMP15 signaling in target cells (Demars et al. 2013). In both models, a lowering of the biological activity of BMP15 is expected, due to lower amounts of the mature protein (as hypothesized in R/+ follicles) or lower functional interactions between BMP15 and GDF9 (as hypothesized in Gr/Gr follicles), but the molecular mechanisms affecting BMP15 are not yet fully understood. Alternatively, the in vivo expression level of each component of the inhibin/activin system could depend on the specific equilibrium of the different components existing within each breed. This hypothesis is supported by the observation that the granulosa cells of non-mutant Grivette ewes expressed higher levels of INHA, INHBA, ACVR1B and ACVR2A, but lower levels of FST and TGFBR3 mRNA than the granulosa cells of non-mutant Rasa Aragonesa ewes. Consistently, the concentrations of activin A and inhibin A proteins were higher in the follicles of Grivette ewes, also illustrating pre-existing differences in the inhibin/activin system between breeds, in the absence of BMP15 mutation.

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

In conclusion, the presence of a loss-of-function BMP15 mutation, such as the FecX<sup>e</sup> mutation when present at the heterozygous state, or the FecX<sup>c</sup>-mutation when present at the homozygous state, is associated with a clear increase in ovulation rate leading to a higher prolificacy. In this study, we have challenged a potential role of the ovarian inhibin/activin system in participating in this phenotype. From our in vitro results, the genes encoding different components of the inhibin/activin system such as INHA, INHBA, FST and TGFBR3 are potential targets of BMP15 in ovine granulosa cells. However, in vivo, the presence of a loss-of-function BMP15 mutation in ewes was not found associated with strong changes in the expression of these components in granulosa cells. Moreover, the observed expression changes were dependent on either the BMP15 mutation itself or the ovine breed in which the mutation was present. Altogether, these results suggest that the ovarian inhibin/activin system is not a key component of the mechanisms regulating the ovulation rate in BMP15 mutant ewes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-16-0507.

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

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