REPRODUCTION
RESEARCH

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

Anthony Estienne[1], Belén Lahoz[2], Peggy Jarrier[1], Loys Bodin[3], José Folch[2], José-Luis Alabart[2], Stéphane Fabre[3] and Danielle Monniaux[1]

[1]UMR85 PRC, INRA, CNRS, IFCE, Université de Tours, Nouzilly, France, [2]Unidad 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 [3]GenPhySE, Université de Toulouse, INRA, INPT, ENVT, 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 $FecX^R$ and the $FecX^{Gr}$ 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 *INHA*, *INHBA* 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.
Reproduction (2017) **153** 395–404

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.* 2005*b*, 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 & Glister 2006), has also been proposed to participate in this mechanism.

© 2017 Society for Reproduction and Fertility
ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-16-0507
Online version via www.reproduction-online.org

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 $FecL^{L}$, mutation which induces an ectopic expression of the *B4GALNT2* (*beta-1,4-N-acetyl-galactosaminyl 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.* 2005*a*). 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 $BMP15^{W154NFsX55}/FecX^{R}$ 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 $BMP15^{T317I}/FecX^{Gr}$ 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 $FecX^{R}$ 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 $FecX^{Gr}$ 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 studies, the stage of follicular 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 (C_t) 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 $R = 100 \times (E_{RPL19}{}^{Ct\ (RPL19)}/E_{target}{}^{Ct\ (target)})$. *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 1/10,000 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

Table 1 Quantitative PCR primers sequences and their efficiency.

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

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

Breed	Rasa Aragonesa		Grivette	
Genotype (No. of ewes)	+/+ (n=6)	R/+ (n=6)	+/+ (n=5)	Gr/Gr (n=5)
No. of small follicles (1–3 mm)	43.0±7.4	42.8±6.5	48.2±3.7	56.6±6.8
No. of large follicles (larger than 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***

*P<0.05, **P<0.01, ***P<0.001 vs +/+ ewes of the same breed.

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).

Ovarian expression of inhibin A and activin A in the Rasa Aragonesa and Grivette breeds and effects of BMP15 mutations

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

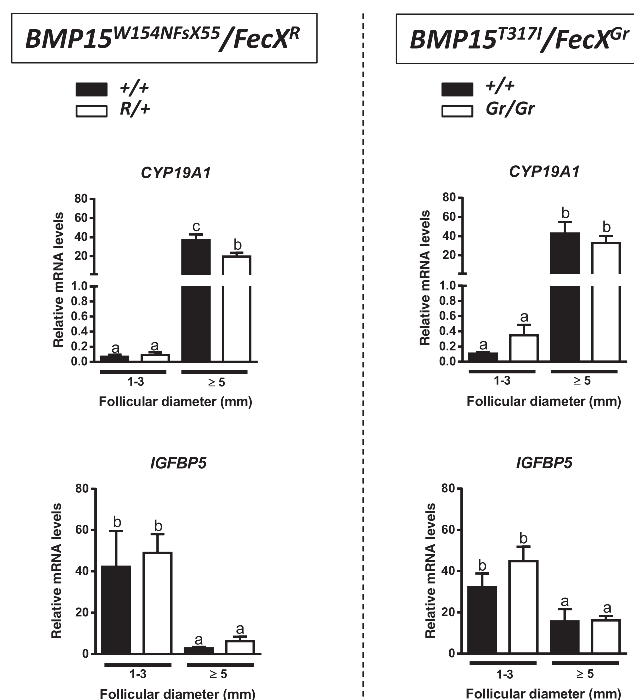

Figure 1 Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of functional gene markers in the granulosa cells of ewes. Results represent *CYP19A1* (a marker of fully differentiated granulosa cells, upper panels) and *IGFBP5* (a marker of apoptotic 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.

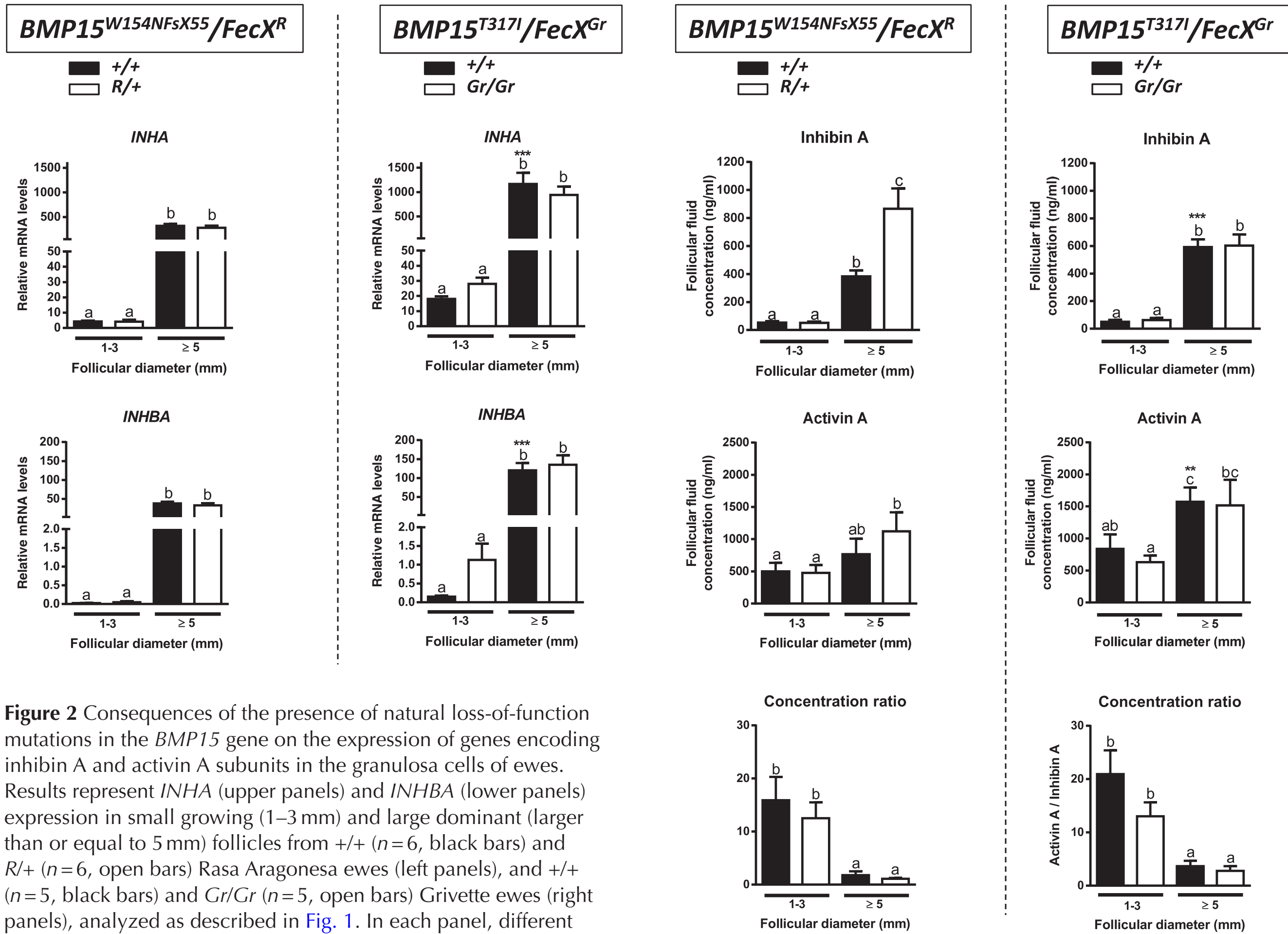

Figure 2 Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding inhibin A and activin A subunits in the granulosa cells of ewes. Results represent *INHA* (upper panels) and *INHBA* (lower panels) expression in small growing (1–3 mm) 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.

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/inhibin A 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/inhibin A 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 concentration of inhibin A in *R*/+, compared to +/+ large follicles, in the Rasa Aragonesa breed ($P<0.05$, Fig. 3).

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 $P<0.001$

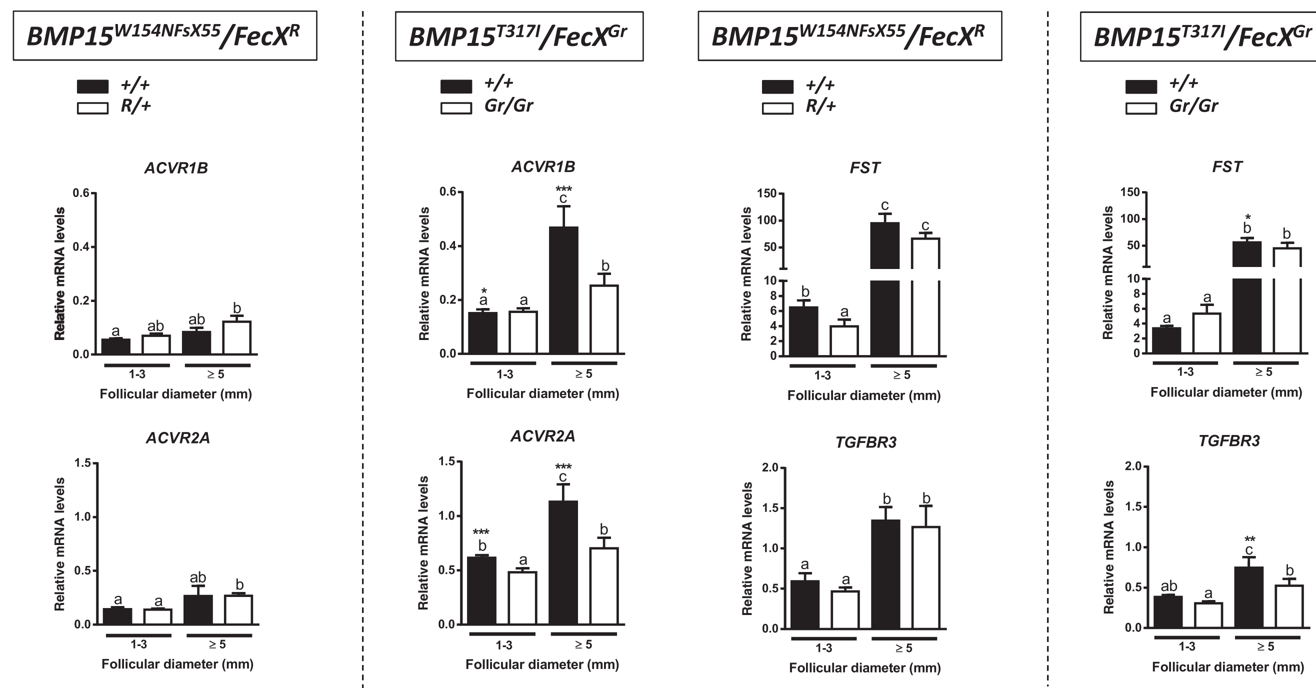

Figure 4 Consequences of the presence of natural loss-of-function mutations in the *BMP15* gene on the expression of genes encoding activin receptors in the granulosa cells of ewes. Results represent *ACVR1B* (upper panels) and *ACVR2A* (lower panels) expression in small growing (1–3 mm) and large dominant (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 < 0.05, ***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 (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 < 0.05, **P < 0.01 vs the corresponding follicle class in +/+ Rasa Aragonesa ewes.

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

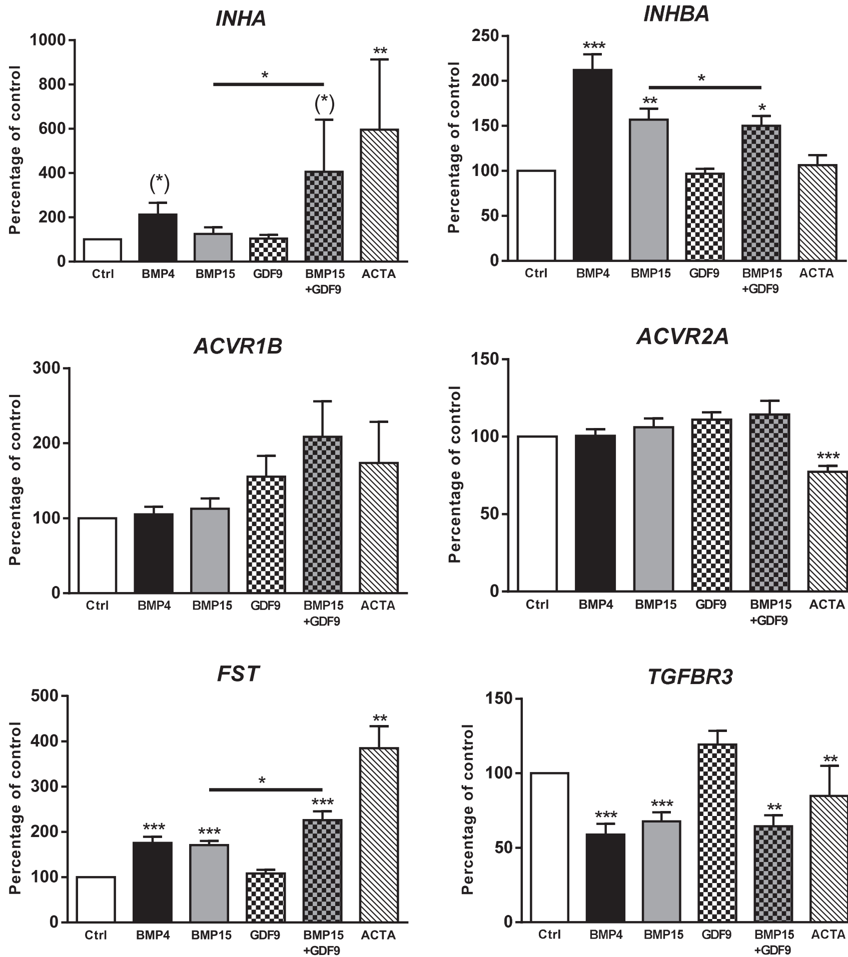

Figure 6 Effect of BMP4, BMP15, GDF9 and activin A on the expression of genes encoding different members of the inhibin/activin system in ovine granulosa cells. Sheep granulosa cells were cultured in 96-well plates for 48 h with or without 50 ng/mL of the different factors. Each treatment was tested 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.

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 48 h-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* ($P<0.01$) and 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 *INHBA* mRNA levels but inhibited *ACVR2A* ($P<0.01$) 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 $FecX^R$ 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 $FecX^{Gr}$ 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 $FecX^I$ mutation in *BMP15* (McNatty *et al.* 2009) and more strikingly for the Booroola ewes carrying the $FecB^B$ mutation in *BMPR1B* (Henderson *et al.* 1985). However, there was no clear effect of the $FecX^R$ and the $FecX^{Gr}$ 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 $FecX^{Gr}$, compared to non-carrier Grivette ewes, but such differences were not observed between the heterozygous $FecX^R$ 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 $FecX^R$ mutation is known to preclude BMP15 production (Martinez-Royo *et al.* 2008), and the $FecX^{Gr}$ 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 expressional 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 $FecX^{R}$ mutation when present at the heterozygous state, or the $FecX^{Gr}$ 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 expressional 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.

Funding

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

Acknowledgements

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

References

Besnard N, Pisselet C, Monniaux D, Locatelli A, Benne F, Gasser F, Hatey F & Monget P 1996 Expression of messenger ribonucleic acids of insulin-like growth factor binding protein-2, -4, and -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. *Biology of Reproduction* **55** 1356–1367. (doi:10.1095/biolreprod55.6.1356)

Bonnet A, Bevilacqua C, Benne F, Bodin L, Cotinot C, Liaubet L, Sancristobal M, Sarry J, Terenina E, Martin P, *et al.* 2011 Transcriptome profiling of sheep granulosa cells and oocytes during early follicular development obtained by laser capture microdissection. *BMC Genomics* **12** 417. (doi:10.1186/1471-2164-12-417)

Demars J, Fabre S, Sarry J, Rossetti R, Gilbert H, Persani L, Tosser-Klopp G, Mulsant P, Nowak Z, Drobik W, *et al.* 2013 Genome-wide association studies identify two novel BMP15 mutations responsible for an atypical hyperprolificacy phenotype in sheep. *PLoS Genetics* **9** e1003482. (doi:10.1371/journal.pgen.1003482)

Drouilhet L, Mansanet C, Sarry J, Tabet K, Bardou P, Woloszyn F, Lluch J, Harichaux G, Viguie C, Monniaux D, *et al.* 2013 The highly prolific phenotype of Lacaune sheep is associated with an ectopic expression of the B4GALNT2 gene within the ovary. *PLoS Genetics* **9** e1003809. (doi:10.1371/journal.pgen.1003809)

Drummond AE, Findlay JK & Ireland JJ 2004 Animal models of inhibin action. *Seminars in Reproductive Medicine* **22** 243–252. (doi:10.1055/s-2004-831900)

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ & Matzuk MM 1998 The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Molecular Endocrinology* **12** 1809–1817. (doi:10.1210/mend.12.12.0206)

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, *et al.* 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* **142** 4891–4899. (doi:10.1210/en.142.11.4891)

Estienne A, Pierre A, di Clemente N, Picard JY, Jarrier P, Mansanet C, Monniaux D & Fabre S 2015 Anti-Mullerian hormone regulation by the bone morphogenetic proteins in the sheep ovary: deciphering a direct regulatory pathway. *Endocrinology* **156** 301–313. (doi:10.1210/en.2014-1551)

Fabre S, Pierre A, Mulsant P, Bodin L, Di Pasquale E, Persani L, Monget P & Monniaux D 2006 Regulation of ovulation rate in mammals: contribution of sheep genetic models. *Reproductive Biology and Endocrinology* **4** 20. (doi:10.1186/1477-7827-4-20)

Forage RG, Brown RW, Oliver KJ, Atrache BT, Devine PL, Hudson GC, Goss NH, Bertram KC, Tolstoshev P, Robertson DM, *et al.* 1987 Immunization against an inhibin subunit produced by recombinant DNA techniques results in increased ovulation rate in sheep. *Journal of Endocrinology* **114** R1–R4. (doi:10.1677/joe.0.114R001)

Galloway SM, Gregan SM, Wilson T, McNatty KP, Juengel JL, Ritvos O & Davis GH 2002 Bmp15 mutations and ovarian function. *Molecular and Cellular Endocrinology* **191** 15–18. (doi:10.1016/S0303-7207(02)00047-3)

Harris RA, Tardif SD, Vinar T, Wildman DE, Rutherford JN, Rogers J, Worley KC & Aagaard KM 2014 Evolutionary genetics and implications of small size and twinning in callitrichine primates. *PNAS* **111** 1467–1472. (doi:10.1073/pnas.1316037111)

Henderson KM, Franchimont P, Lecomte-Yerna MJ, Hudson N & Ball K 1984 Increase in ovulation rate after active immunization of sheep with inhibin partially purified from bovine follicular fluid. *Jornal of Endocrinology* **102** 305–309. (doi:10.1677/joe.0.1020305)

Henderson KM, Kieboom LE, McNatty KP, Lun S & Heath D 1985 Gonadotrophin-stimulated cyclic AMP production by granulosa cells from Booroola x Romney ewes with and without a fecundity gene. *Journal of Reproduction and Fertility* **75** 111–120. (doi:10.1530/jrf.0.0750111)

Hillard MA, Wilkins JF, Cummins LJ, Bindon BM, Tsonis CG, Findlay JK & O'Shea T 1995 Immunological manipulation of ovulation rate for twinning in cattle. In *Reproduction in Domestic Ruminants III:*

proceedings of the Fourth International Symposium on Reproduction in Domestic Ruminants. Eds RJ Scaramuzzi, CD Nancarrow & C Doberska. Townsville, Australia; 2–6 October 1994. *Journal of Reproduction and Fertility* Supplement **49** 351–364.

Hoekstra C, Zhao ZZ, Lambalk CB, Willemsen G, Martin NG, Boomsma DI & Montgomery GW 2008 Dizygotic twinning. *Human Reproduction Update* **14** 37–47. (doi:10.1093/humupd/dmm036)

Knight PG & Glister C 2006 TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191–206. (doi:10.1530/rep.1.01074)

Knight PG, Satchell L & Glister C 2012 Intra-ovarian roles of activins and inhibins. *Molecular and Cellular Endocrinology* **359** 53–65. (doi:10.1016/j.mce.2011.04.024)

Lahoz B, Alabart JL, Jurado JJ, Calvo JH, Martinez-Royo A, Fantova E & Folch J 2011 Effect of the FecX(R) polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application. *Journal of Animal Science* **89** 3522–3530. (doi:10.2527/jas.2010-3828)

Li Q, Rajanahally S, Edson MA & Matzuk MM 2009 Stable expression and characterization of N-terminal tagged recombinant human bone morphogenetic protein 15. *Molecular Human Reproduction* **15** 779–788. (doi:10.1093/molehr/gap062)

Liao WX, Moore RK, Otsuka F & Shimasaki S 2003 Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. *Journal of Biological Chemistry* **278** 3713–3719. (doi:10.1074/jbc.M210598200)

Logan KA, Juengel JL & McNatty KP 2002 Onset of steroidogenic enzyme gene expression during ovarian follicular development in sheep. *Biology of Reproduction* **66** 906–916. (doi:10.1095/biolreprod66.4.906)

Luong HT, Chaplin J, McRae AF, Medland SE, Willemsen G, Nyholt DR, Henders AK, Hoekstra C, Duffy DL, Martin NG, *et al.* 2011 Variation in BMPR1B, TGFRB1 and BMPR2 and control of dizygotic twinning. *Twin Research and Human Genetics* **14** 408–416. (doi:10.1375/twin.14.5.408)

Martinez-Royo A, Jurado JJ, Smulders JP, Marti JI, Alabart JL, Roche A, Fantova E, Bodin L, Mulsant P, Serrano M, *et al.* 2008 A deletion in the bone morphogenetic protein 15 gene causes sterility and increased prolificacy in Rasa Aragonesa sheep. *Animal Genetics* **39** 294–297. (doi:10.1111/j.1365-2052.2008.01707.x)

Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskyi O, Ritvos O & Hsueh AJ 2004 Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Molecular Endocrinology* **18** 653–665. (doi:10.1210/me.2003-0393)

Mbarek H, Steinberg S, Nyholt DR, Gordon SD, Miller MB, McRae AF, Hottenga JJ, Day FR, Willemsen G, de Geus EJ, *et al.* 2016 Identification of common genetic variants influencing spontaneous dizygotic twinning and female fertility. *American Journal of Human Genetics* **98** 898–908. (doi:10.1016/j.ajhg.2016.03.008)

McGrath SA, Esquela AF & Lee SJ 1995 Oocyte-specific expression of growth/differentiation factor-9. *Molecular Endocrinology* **9** 131–136. (doi:10.1210/mend.9.1.7760846)

McIntosh CJ, Lun S, Lawrence S, Western AH, McNatty KP & Juengel JL 2008 The proregion of mouse BMP15 regulates the cooperative interactions of BMP15 and GDF9. *Biology of Reproduction* **79** 889–896. (doi:10.1095/biolreprod.108.068163)

McNatty KP, Juengel JL, Wilson T, Galloway SM, Davis GH, Hudson NL, Moeller CL, Cranfield M, Reader KL, Laitinen MP, *et al.* 2003 Oocyte-derived growth factors and ovulation rate in sheep. In *Reproduction in Domestic Ruminants V: proceedings of the Sixth International Symposium on Reproduction in Domestic Ruminants*. Eds BK Campbell *et al*. Crieff, Scotland, UK; August 2002. *Reproduction* Supplement **61** 339–351.

McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa S, Lawrence SB, Western A, Meerasahib MF, Mottershead DG, Groome NP, *et al.* 2005*a* Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. *Reproduction* **129** 473–480. (doi:10.1530/rep.1.0511)

McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa S, Lawrence SB, Western A, Meerasahib MF, Mottershead DG, Groome NP, *et al.* 2005*b* Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. *Reproduction* **129** 481–487. (doi:10.1530/rep.1.00517)

McNatty KP, Heath DA, Hudson NL, Lun S, Juengel JL & Moore LG 2009 Gonadotrophin-responsiveness of granulosa cells from bone morphogenetic protein 15 heterozygous mutant sheep. *Reproduction* **138** 545–551. (doi:10.1530/REP-09-0154)

Monestier O, Servin B, Auclair S, Bourquard T, Poupon A, Pascal G & Fabre S 2014 Evolutionary origin of bone morphogenetic protein 15 and growth and differentiation factor 9 and differential selective pressure between mono- and polyovulating species. *Biology of Reproduction* **91** 83. (doi:10.1095/biolreprod.114.119735)

Monniaux D, di Clemente N, Touzé JL, Belville C, Rico C, Bontoux M, Picard JY & Fabre S 2008 Intrafollicular steroids and anti-mullerian hormone during normal and cystic ovarian follicular development in the cow. *Biology of Reproduction* **79** 387–396. (doi:10.1095/biolreprod.107.065847)

Montgomery GW, Galloway SM, Davis GH & McNatty KP 2001 Genes controlling ovulation rate in sheep. *Reproduction* **121** 843–852. (doi:10.1530/rep.0.1210843)

Moore RK & Shimasaki S 2005 Molecular biology and physiological role of the oocyte factor, BMP-15. *Molecular and Cellular Endocrinology* **234** 67–73. (doi:10.1016/j.mce.2004.10.012)

Moore RK, Otsuka F & Shimasaki S 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *Journal of Biological Chemistry* **278** 304–310. (doi:10.1074/jbc.M207362200)

Mottershead DG, Sugimura S, Al-Musawi SL, Li JJ, Richani D, White MA, Martin GA, Trotta AP, Ritter LJ, Shi J, *et al.* 2015 Cumulin, an oocyte-secreted heterodimer of the transforming growth factor-beta family, is a potent activator of granulosa cells and improves oocyte quality. *Journal of Biological Chemistry* **290** 24007–24020. (doi:10.1074/jbc.M115.671487)

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF & Shimasaki S 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. *Journal of Biological Chemistry* **275** 39523–39528. (doi:10.1074/jbc.M007428200)

Otsuka F, Yamamoto S, Erickson GF & Shimasaki S 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *Journal of Biological Chemistry* **276** 11387–11392. (doi:10.1074/jbc.M010043200)

Palmer JS, Zhao ZZ, Hoekstra C, Hayward NK, Webb PM, Whiteman DC, Martin NG, Boomsma DI, Duffy DL & Montgomery GW 2006 Novel variants in growth differentiation factor 9 in mothers of dizygotic twins. *Journal of Clinical Endocrinology and Metabolism* **91** 4713–4716. (doi:10.1210/jc.2006-0970)

Persani L, Rossetti R, Di Pasquale E, Cacciatore C & Fabre S 2014 The fundamental role of bone morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. *Human Reproduction Update* **20** 869–883. (doi:10.1093/humupd/dmu036)

Pierre A, Estienne A, Racine C, Picard JY, Fanchin R, Lahoz B, Alabart JL, Folch J, Jarrier P, Fabre S, *et al.* 2016 The bone morphogenetic protein 15 up-regulates the anti-mullerian hormone receptor expression in granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **101** 2602–2611. (doi:10.1210/jc.2015-4066)

Tannetta DS, Feist SA, Bleach EC, Groome NP, Evans LW & Knight PG 1998 Effects of active immunization of sheep against an amino terminal peptide of the inhibin alpha C subunit on intrafollicular levels of activin A, inhibin A and follistatin. *Journal of Endocrinology* **157** 157–168. (doi:10.1677/joe.0.1570157)

Visser JA & Themmen AP 2005 Anti-Mullerian hormone and folliculogenesis. *Molecular and Cellular Endocrinology* **234** 81–86. (doi:10.1016/j.mce.2004.09.008)

Visser JA & Themmen AP 2014 Role of anti-Mullerian hormone and bone morphogenetic proteins in the regulation of FSH sensitivity. *Molecular and Cellular Endocrinology* **382** 460–465. (doi:10.1016/j.mce.2013.08.012)

Walton KL, Makanji Y & Harrison CA 2012 New insights into the mechanisms of activin action and inhibition. *Molecular and Cellular Endocrinology* **359** 2–12. (doi:10.1016/j.mce.2011.06.030)

Yan L, Li H & Shi Z 2015 Immunization against inhibin improves in vivo and in vitro embryo production. *Animal Reproduction Science* **163** 1–9. (doi:10.1016/j.anireprosci.2015.11.001)

Received 14 September 2016
First decision 20 October 2016
Revised manuscript received 30 November 2016
Accepted 9 January 2017