The bone morphogenetic protein 15 up-regulates the anti-Müllerian hormone receptor expression in granulosa cells

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Context: Anti-Müllerian hormone (AMH) is produced by the granulosa cells (GCs) of growing follicles and inhibits follicular development.

Objective: This study aimed to investigate the regulation of the AMH-specific receptor gene (*AMHR2*) expression in GCs by bone morphogenetic protein 15 (BMP15), BMP4 and growth differentiation factor 9 (GDF9).

Design, Setting, Patients: Their effects on *AMHR2* and *AMH* mRNAs were studied in luteinized human GCs (hGCs) and in ovine GCs (oGCs) from small antral follicles. The effects of BMPs on human *AMHR2* and *AMH* promoter reporter activities were analyzed in transfected oGCs. The *in vivo* effect of BMP15 on GCs *AMHR2* and *AMH* expression was investigated by using Lacaune and Rasa Aragonesa hyperprolific ewes carrying loss-of-function mutations in *BMP15*.

Main Outcome Measures: mRNAs were quantified by real-time RT-PCR. Promoter reporter constructs activities were quantified by the measurement of their luciferase activity.

Results: BMP15 and BMP4 enhanced *AMHR2* and *AMH* expression in hGCs and in oGCs whereas GDF9 had no effect. In oGCs, GDF9 increased BMP15 effect on *AMH* expression. Consistent with these results, BMP15 and BMP4, but not GDF9, enhanced *AMHR2* promoter activity in oGCs, whereas GDF9 increased BMP15 effect on *AMH* promoter activity. Moreover, oGCs from both BMP15 mutant ewes had reduced *AMHR2* mRNA levels but unchanged *AMH* expression compared to wild-type ewes.

Conclusions: Altogether, these results suggest that the mechanisms of action of BMP15 on *AMHR2* and *AMH* expression are different, and that by stimulating *AMHR2* and *AMH* expression in GCs BMP15 enhances AMH inhibitory actions in GCs.

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Abbreviations:

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance, is a member of the transforming growth factor beta (TGFß) family well known for its role on Müllerian duct regression during male sexual differentiation (1). In females, *AMH* is expressed in the granulosa cells (GCs) of growing follicles and it importantly modulates follicle growth and maturation, particularly by inhibiting the FSH sensitivity of follicles (2). Serum AMH is increasingly used in clinical practice as an early indicator of GCs tumors (3), as well as a reliable marker of ovarian reserve and of ovarian response to gonadotropin-based stimulatory treatments (4). The expression of *AMH* starts in primary follicles, is the highest in small antral ones and then decreases as follicles grow, except in cumulus cells surrounding the oocytes (5). In rat (6) and rabbit (7) ovaries, the AMH-specific type II receptor *AMHR2* is coexpressed with *AMH* in GCs from growing preantral and small antral follicles. In human preovulatory follicles, both *AMH* and *AMHR2* mRNAs are more expressed in cumulus cells than in mural GCs (8). However, the factors involved in the expression pattern of *AMH* and *AMHR2* in GCs are still poorly documented.

The bone morphogenetic proteins (BMPs) are cytokines also belonging to the TGFß family, and over 20 BMPs have been identified up to now. BMPs contribute to the regulation of cellular proliferation, differentiation and apoptosis in a variety of tissues, including the ovary in which they are important regulators of folliculogenesis. Particularly, BMP15 and GDF9, known to be specifically expressed in the oocyte (9, 10) and acting alone or in synergy, are key regulators of follicle development, ovulation rate and oocyte quality (11–14). BMP4 is detected in granulosa and/or theca follicular cells depending upon species (15) and it modulates gonadotropin-induced steroidogenesis (16, 17). BMP15 and BMP4 activate SMAD1/5/8 signaling pathway whereas GDF9 and activin signal through SMAD2/3 proteins (18, 19). Recently, several BMPs have been shown to enhance *AMH* expression in GCs (20–24), but their effect on *AMHR2* expression is unknown.

The aim of this work was to test whether BMPs could regulate *AMHR2* expression and its promoter activity. The effects of oocyte-derived BMP15 and GDF9 were compared with those of granulosa/theca cells-derived BMP4, a canonical inducer of the BMP signaling pathway. The regulation of *AMHR2* mRNAs was studied in vitro in two models of primary GCs at different stages of their differentiation: human granulosa-luteal cells (hGCs) from women undergoing assisted reproduction technology (ART) procedures and ovine GCs (oGCs) from small antral follicles. The mechanism of BMP15, GDF9 and BMP4 action on *AMHR2* promoter was addressed in oGCs. In addition, we analyzed the in vivo effect on GCs *AMHR2* expression of two natural loss-of-function mutations of *BMP15* carried by Lacaune and Rasa Aragonesa ewes, which cause hyperprolificacy when present at the heterozygous state and sterility when present at the homozygous state (25, 26).

Materials and Methods

Subjects

Twenty-nine patients aged 24–38 years undergoing ART were included in this study according to the following inclusion criteria: 1) both ovaries present, with no morphological abnormalities at transvaginal ultrasound; 2) menstrual cycle length range between 25 and 35 days; 3) no current or past diseases affecting ovaries or gonadotropin or sex steroid secretion, clearance, or excretion; 4) no clinical signs of hyperandrogenism; 5) body mass indexes ranging between 18 and 25 kg/m^2, and 6) inclusion in a standard long GnRH agonist suppression protocol. An informed consent was obtained from all women, and this investigation received the approval of our internal institutional review board (IRB).

Collection and culture of human granulosa-luteal cells

After oocyte isolation, follicular fluids from each patient were centrifuged as described (27) through a Percoll gradient at 350 *g* for 15 minutes. Human GCs were collected at the interface and seeded at 3×10^5 cells per well in 6-well plates in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all products from Invitrogen, Carlsbad, CA). The next day, hGCs were treated for 48 hours in serum-free conditions with or without 10 ng/ml of recombinant human (rh) BMP4, BMP15 and/or GDF9 (R&D Systems Europe, Lille, France).

Animals

For cultures of oGCs, ovaries were recovered from ewes of various breeds during the period of seasonal reproduction after slaughtering in a local slaughterhouse at Nouzilly (France). For in vivo *AMHR2* expression studies, ovaries from all genotypes were recovered in the follicular phase of the cycle as described (20), after slaughtering of the ewes at Zaragoza (Spain) and Toulouse (France), for the Rasa Aragonesa and Lacaune 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 ES 50 297 0012 005 in Zaragoza) in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Collection and culture of ovine 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 when useful and oGCs were recovered as described (20).

For culture experiments, oGCs from 1–3 mm follicles were cultured for 48 hours in serum-free conditions (20) with and

without rh BMP4, human BMP15, mouse GDF9 or human/mouse/rat Activin A (50 ng/ml each; R&D System Europe).

For the evaluation of in vivo *AMHR2* and *AMH* expression, three follicular classes were defined (1–3 mm; 3–5 mm; >5 mm) corresponding to follicle growth, selection and dominance stages, respectively. Then oGC pools of each follicle size-class were established per animal.

Western blotting analysis

Twenty-four hours after seeding, for hGCs, and immediately after collection, for oGCs, cells were serum starved, treated 1 hour with human BMP4, BMP15, GDF9, both BMP15 and GDF9, or Activin A (10 ng/ml or 50 ng/ml each for hGCs and oGCs respectively) and lysates were prepared as described (27). Thirty μg were subjected to SDS-PAGE (Bio-Rad, Marnes-la-Coquette, France), then blots were successively incubated with antibodies against phospho-SMAD/1/5/8, phospho-SMAD/2/3 (Cell Signaling Technology, Ozyme, Montigny-le-Bretonneux, France) both at 1:1000, and Actin (Sigma, L'Isle d'Abeau Chesnes, France). Then membranes were probed with peroxidase-labeled antibodies (1:5000) and the bands were visualized with the ECL Plus detection reagent (Pierce, ThermoFischer Scientific, Villebon-sur-Yvette, France).

RNA extraction and quantitative real-time PCR

Total RNAs were extracted from hGCs using the RNA Plus extraction kit (Qiagen, Courtaboeuf, France) and from oGCs using the Nucleospin RNA II kit (Macherey-Nagel, Hoerdt, France). Reverse transcription was performed with the Omniscript Reverse Transcription Kit (Qiagen) using 1 μg RNA.

Quantification of *AMHR2*, *AMH*, *STAR* (Steroidogenic Acute Regulatory protein), *CYP19A1* (Cytochrome P450, Family 19, Subfamily A, Polypeptide 1/Aromatase) and *RPL19* (Ribosomal Protein L19) mRNAs in hGCs was performed by real-time PCR using the TaqMan PCR method (Roche Diagnostics, Meylan, France) as described (27) with the primers and probes shown in Supplemental Table 1. For oGCs, *AMHR2*, *AMH*, *SMAD6, CYP11A1* (Cytochrome P450, Family 11, Subfamily A, Polypeptide 1/P450 side chain cleavage enzyme) and *RPL19* mRNAs were quantified using SYBR Green Supermix on an iCycler iQ multicolor detection system (Bio-Rad) as previously described (28) with the primers shown in Supplemental Table 1. The cycle threshold (Ct) of the target gene was compared with that of the *RPL19* internal reference (Ref) gene, according to the ratio $R = [E_{Ref}{}^{Ct\ (Ref)}/E_{target}{}^{Ct\ (target)}]$.

Gene constructs, targeted mutagenesis and transient transfection studies

A 2252 (-2197,+54) base pairs (bp) human *AMHR2* promoter (RefSeqGene NG_015981) reporter construct (2252_AMHR2-luc) was prepared by cloning successively a 428 bp (-374,+54) fragment and a 1825 bp distal promoter fragment (-2199,-374) into the BglII and HindIII sites of the pGL2-Basic vector.

Ovine GCs were seeded in 24-well plates and reporter gene constructs (750 ng/well) were transfected into oGCs for 6 hours using Lipofectamine PLUS transfection reagent (ThermoFisher Scientific) as described (20). After transfection, cells were cultured for 24 hours in serum-free conditions with or without rh BMP4 (50 ng/ml), human BMP15 (100 ng/ml), mouse GDF9 (100 ng/ml) or a combination of BMP15 and GDF9 before luciferase assay (Luciferase Assay System, Promega, Charbonnières, France).

AMHR2 immunohistochemistry

Two ovaries of each genotype were recovered from Lacaune and Rasa Aragonesa ewes, fixed in Bouin, embedded in paraffin and serially sectioned at a thickness of 7 μm. Immunohistochemistry analyses were performed as described (22) using a mouse monoclonal antibody raised against human AMHR2 (antibody 12G4, 1/200, (29).

Statistics

All data were analyzed using the Prism 6 Software (GraphPad Software Inc., La Jolla, CA, USA) and the tests mentioned in the figure legends. Results are presented as mean ± SEM.

Results

AMHR2 expression is increased by BMP4 and BMP15 in human GCs

The regulation of *AMHR2* expression by BMP4, BMP15 and GDF9 was analyzed in hGCs obtained from women undergoing ART. First, we checked the ability of these cells to respond to BMP family members. As expected, BMP4 or BMP15 induced the phosphorylation of the SMAD1/5/8 signaling proteins whereas GDF9 induced that of SMAD2/3, the combination of BMP15 and GDF9 activating both signaling pathways (Figure 1A). Then, we tested the effects of a 48 hours treatment by BMPs on the expression of some of their known target genes and of *AMHR2* in hGCs (Figure 1B, C, D, E). BMP4 inhibited *STAR* expression, had no effect on *CYP19A1* and enhanced *AMH* mRNA levels. BMP15 inhibited *STAR* expression whereas it up-regulated *CYP19A1* and *AMH* mRNA levels. GDF9 decreased *CYP19A1* expression but had no effect on *STAR* and *AMH* mRNAs levels. The combination of BMP15 and GDF9 induced a more pronounced decrease in *STAR* and *CYP19A1* mRNA levels than did BMP15 or GDF9 alone, but had the same effect as BMP15 on *AMH* expression. Interestingly *AMHR2* expression was stimulated by both BMP4 and BMP15, whereas GDF9 alone or in combination with BMP15 had no effect (Figure 1E).

AMHR2 expression is increased by BMP4 and BMP15 in ovine GCs

We then studied the regulation of *AMHR2* expression in oGCs from small antral growing follicles, which highly express *AMH*. Here again, we first checked the ability of the cells to respond to BMP family members. We also used Activin A, another member of the TGFß family known to activate the same SMAD2/3 signaling pathway as GDF9 (30). As expected, BMP4 or BMP15 induced the phos-

phorylation of the SMAD1/5/8 signaling proteins whereas GDF9 and Activin A induced that of SMAD2/3, the combination of BMP15 and GDF9 activating both signaling pathways (Figure 2A). Then, we tested the effects of a 48 hours treatment by BMPs and Activin A on the expression of some of their known target genes and of *AMHR2* in oGCs (Figure 2B, C, D, E). *SMAD6* and *CYP11A1* were chosen as BMP-target genes in oGCs instead of *STAR* and *CYP19A1* because at this stage of differentiation these latter genes are poorly expressed by oGCs. BMP4 enhanced *SMAD6, CYP11A1* and *AMH* expression in oGCs. BMP15 also up-regulated *SMAD6, CYP11A1* and *AMH* mRNA levels. GDF9 alone down-regulated *CYP11A1* expression and had no effect on *SMAD6* and

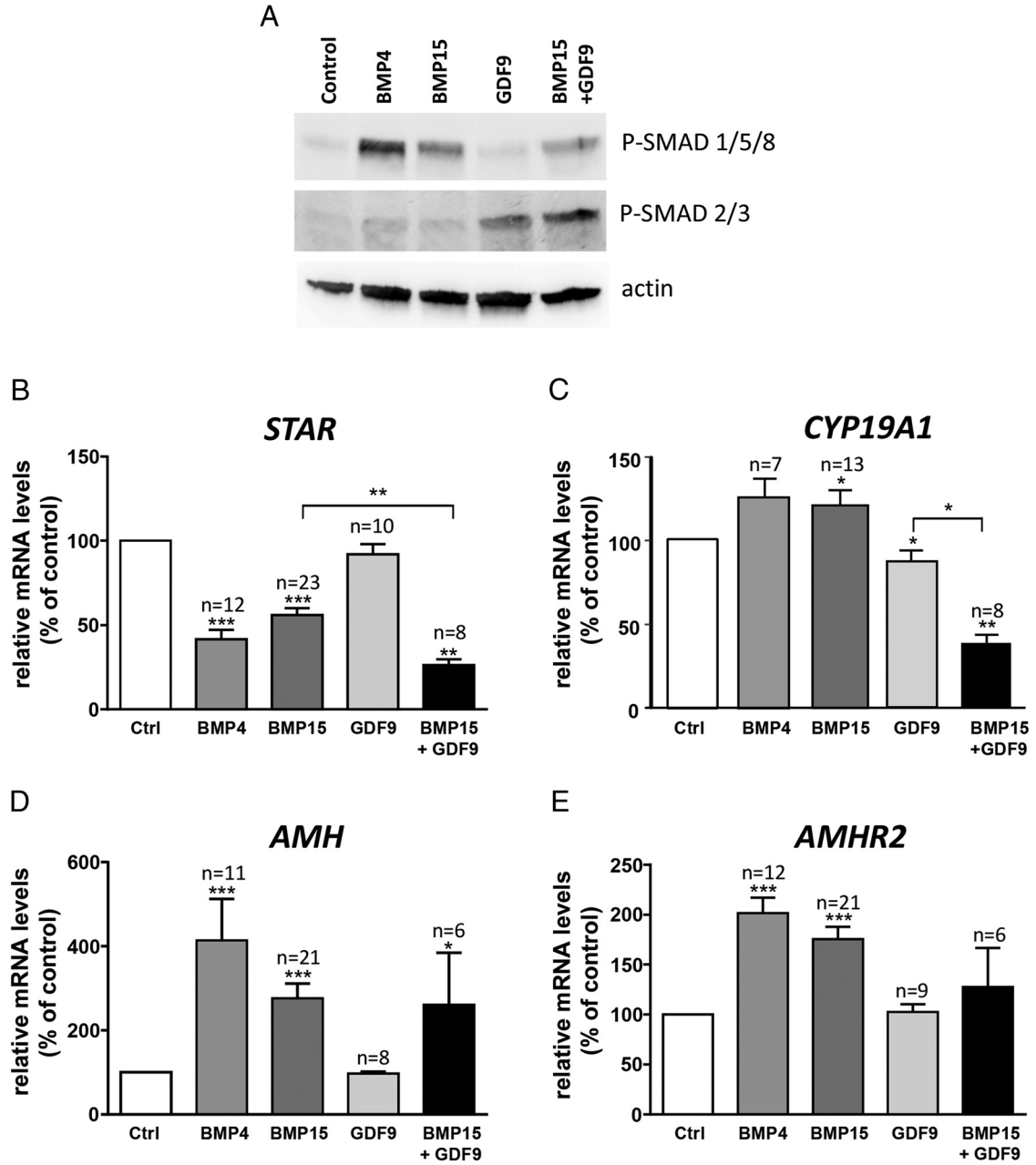


Figure 1. Effect of BMP4, BMP15 and GDF9 on human granulosa cells (hGCs). (A) Western blotting analysis of SMAD phosphorylation in hGCs, after one hour stimulation in control medium or with 10 ng/ml of human BMP4, BMP15, GDF9, or BMP15 + GDF9 (10 ng/ml each). Actin detection was used as gel loading control. (B, C, D, E) Expression of *STAR*, *CYP19A1*, *AMH* and *AMHR2* in hGCs after 48 hours of culture in control medium (Ctrl, cells cultured without ligand) or with human BMP4 (10 ng/ml), BMP15 (10 ng/ml), GDF9 (10 ng/ml) or BMP15 + GDF9 (10 ng/ml each). Messenger RNA accumulation of *STAR* (B), *CYP19A1* (C), *AMH* (D), *AMHR2* (E) 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%). n represents the number of women. Because the small number of cells did not allow us to apply all the different treatments in the same experiment, data were analyzed using the Wilcoxon matched pairs test; *, $P < .05$; **, $P < .01$; ***, $P < .001$, vs Ctrl or between BMP15 and BMP15+GDF9 conditions (black bar) or between GDF9 and BMP15+GDF9 conditions (black bar).

AMH mRNA levels. The combination of BMP15 and GDF9 slightly reduced BMP15 effects on *SMAD6* and *CYP11A1* expression, but enhanced its stimulating effect on *AMH* expression. Activin A inhibited *SMAD6* and *CYP11A1* expression and up-regulated *AMH* mRNA levels. Like in hGCs, *AMHR2* expression was enhanced by BMP4 or BMP15 in oGCs (Figure 2D). GDF9 and Activin A had no effect. The combination of BMP15 and GDF9 had a stimulatory effect comparable to that obtained with BMP15 alone. Similar results were obtained with rh GDF9 instead of murine GDF9 (data not shown).

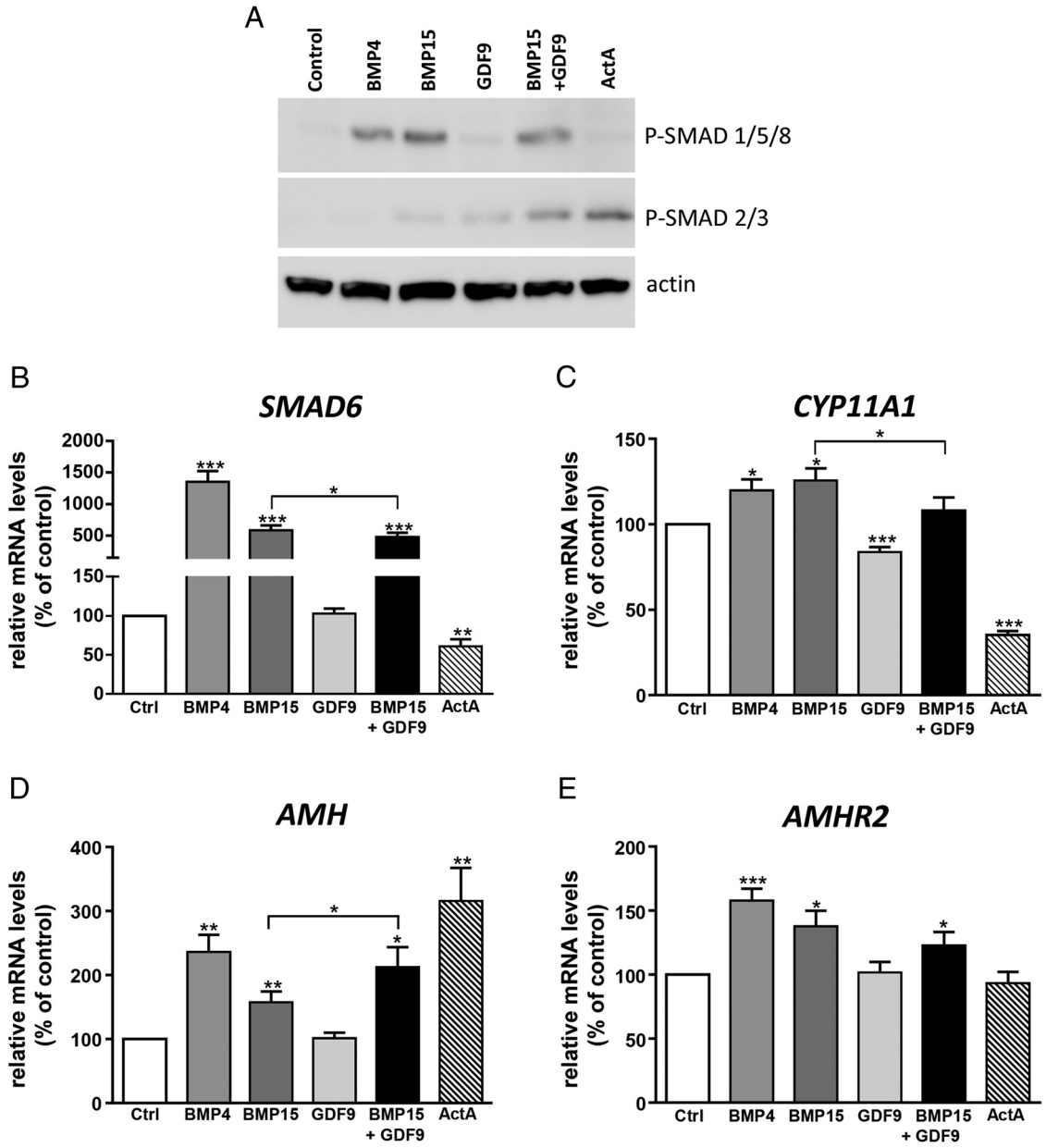
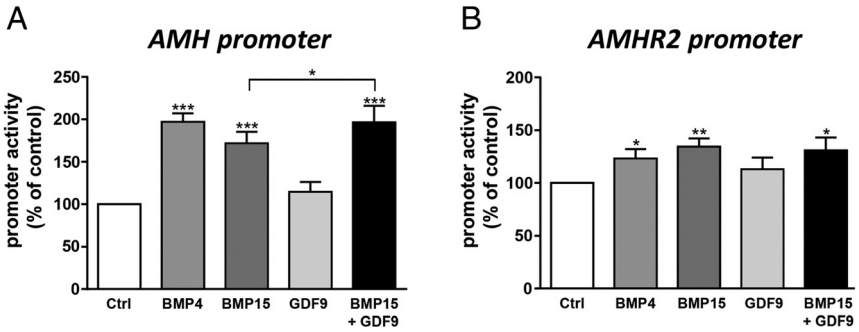


Figure 2. Effect of BMP4, BMP15, GDF9 and Activin A on ovine granulosa cells (oGCs). (A) Western blotting analysis of SMAD phosphorylation in oGCs from 1 to 3 mm follicles, after one hour stimulation in control medium or with 50 ng/ml of human BMP4, BMP15, GDF9, BMP15 + GDF9 (50 ng/ml each) or Activin A (ActA). Actin detection was used as gel loading control. (B, C, D, E) oGCs 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 *SMAD6* (B), *CYP11A1* (C), *AMH* (D) and *AMHR2* (E) 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 10 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, followed by Fisher's LSD test for multiple comparisons.*: $P < .05$, **: $P < .01$, ***: $P < .001$, vs. Ctrl or between BMP15 and BMP15+GDF9 conditions (black bar).



C

Potential SBE/BRE	Sequence
-1354	CACAC
-736	GTCT
-697	CAGGCGCC
-695	GGCGCC
-673	CAGGAGGC
-641	CAGGAGGC
-87	CAGCAGCC

Figure 3. Effect of BMP4, BMP15 and GDF9 on *AMH* and *AMHR2* promoter activities in oGCs. (A, B) Granulosa cells recovered from 1 to 3 mm ovine follicles were seeded in 24-well plates at 250 000 cells/well, then they were transfected with (A) an *AMH* reporter gene construct (consisting of 423 bp from the human *AMH* promoter) or (B) with an *AMHR2* reporter gene construct (consisting of 2252 bp from the human *AMHR2* promoter). Both promoter fragments were inserted upstream of the luciferase reporter gene. After transfection, oGCs were cultured for 24 hours in control medium (Ctrl, cells cultured without ligand) or with human BMP4 (50 ng/ml), human BMP15 (100 ng/ml), mouse GDF9 (100 ng/ml) or BMP15+GDF9 (100 ng/ml each). Each treatment was tested in triplicate. Results represent the mean of six independent experiments and are expressed as a percentage of activity relatively to that of cells cultured without ligand (Ctrl, fixed at 100%). Data were analyzed using two-way ANOVA (in order to estimate the treatment and the culture effect), followed by Fisher's LSD test for multiple comparisons. *: $P < .05$, **: $P < .01$, ***: $P < .001$, vs. Ctrl or between BMP15 and BMP15+GDF9 conditions (black bar). C, Potential SBE and BRE on the human *AMHR2* promoter (relative to +1 transcription start site).

BMP4 and BMP15 stimulate the activity of a human *AMHR2* promoter construct

We next tested whether BMP4, BMP15 and GDF9 could regulate the human *AMHR2* gene promoter activity. Primary cultures of oGCs were transfected with the 2252_AMHR2-luc construct or a human *AMH* proximal promoter construct (423_AMH-luc) used as a control of the BMP responsiveness of the transfected oGCs (20). The activity of the 423_AMH-luc construct was stimulated by BMP4 as previously shown (20), but also by BMP15 and the combination of BMP15 and GDF9, GDF9 alone being ineffective (Figure 3A). The combination of BMP15 and GDF9 slightly enhanced BMP15 effects on 423_AMH-luc activity. BMP4 and BMP15 increased 2252_AMHR2-luc activity (Figure 3B); GDF9 had no effect on this construct and the effect of the combination of BMP15 and GDF9 was similar to the effect of BMP15 alone. Potential SMAD-binding elements (SBEs) and BMP-response elements (BRE) (31) on the 2252 bp human *AMHR2* promoter are indicated in Figure 3C.

AMHR2 expression is reduced in two models of ewes carrying natural mutations in the BMP15 gene

Finally, we took advantage of sheep models carrying natural mutations in the *BMP15* gene to study the in vivo effect of BMP15 on *AMHR2* expression in growing follicles. We analyzed the differential expression level of *AMHR2* and *AMH* mRNA in GCs from Lacaune and Rasa Aragonesa ewes either noncarriers or heterozygous carriers of the $FecX^L$ or the $FecX^R$ mutations, respectively. The $FecX^L$ mutation is a C321Y missense non conservative substitution of a cysteine to a tyrosine in the mature peptide of the protein, which leads to a defect in the protein secretion (26). The $FecX^R$ mutation is a 17 bp deletion which creates a premature stop codon in the proregion (25). Both mutations lead to a total loss of BMP15 function (25, 26). In both breeds, *AMH* mRNAs levels in mutant ewes were not significantly different from those obtained for wild-type animals in all categories of antral follicles (Figure 4A, C). In contrast, *AMHR2* expression was dramatically reduced in GCs from 3–5 mm and > 5 mm in $FecX^L$-carrier Lacaune and 3–5 mm in $FecX^R$-carrier Rasa Aragonesa antral follicles when compared to wild-type follicles (Figure 4B, D). In keeping with these results, AMHR2 immunostaining was lower in GCs of mutant ewes than in wild-type ones (Figure 4E, F and Supplemental Figure 1).

Discussion

In this work, we showed that BMP15 and BMP4 significantly enhanced *AMHR2* mRNA levels in hGCs and in oGCs, whereas GDF9 acting alone or in combination with BMP15 had no effect. In oGCs, BMP15 and BMP4 enhanced also *AMH* mRNA levels, and GDF9 increased the stimulating effect of BMP15. In keeping with these results, BMP15 and BMP4, but not GDF9, enhanced *AMHR2* promoter activity in oGCs, whereas GDF9 increased the stimulating effect of BMP15 on *AMH* promoter activity. Moreover, oGCs from hyperprolific ewes, carrying loss-

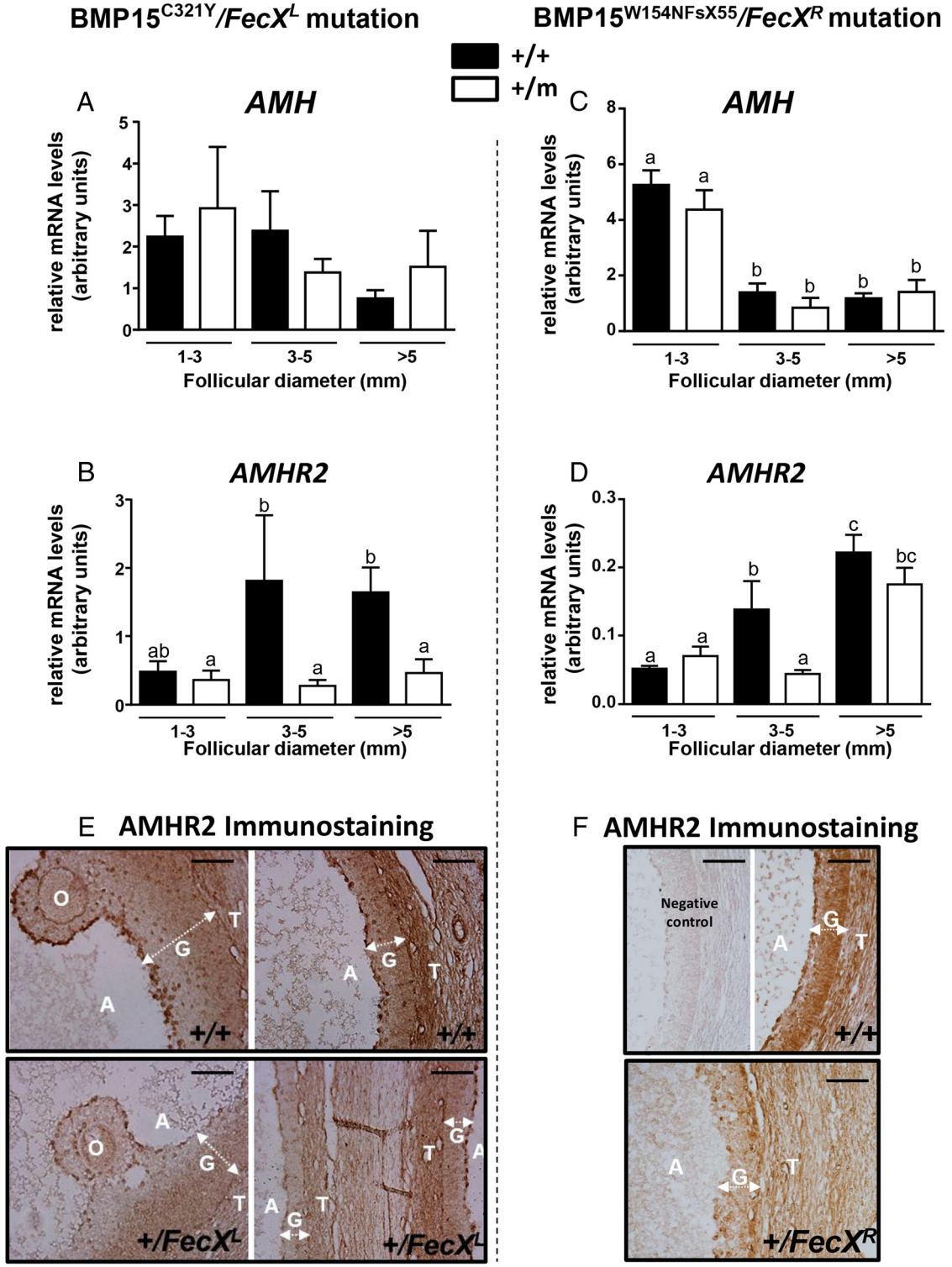


Figure 4. Consequence of the presence of natural loss-of-function mutations in the *BMP15* gene on *AMH* and *AMHR2* expression in oGCs from sheep growing follicles. (A, B, C, D) For each ewe, follicles recovered after ovarian dissection were allocated to 3 size classes (1–3 mm, 3–5 mm, and > 5 mm). In panels A to D, results represent *AMH* and *AMHR2* mRNA expression in granulosa cells recovered from +/*FecX*^{L} (n = 5) and +/+ (n = 7) Lacaune ewes (A and B), and +/*FecX*^{R} (n = 6) and +/+ (n = 6) Rasa Aragonesa ewes (C and D). *AMH* (A and C) and *AMHR2* (B and D) mRNA accumulation was studied by reverse transcription and quantitative PCR and represented as mRNA relative level, using *RPL19* as an internal reference. Data were analyzed by one-way ANOVA. Different letters indicate significant differences ($P < .05$) between follicular classes and genotypes. (E, F) Photomicrographs of ovarian sections from wild-type (+/+) and mutant +/*FecX*^{L} and +/*FecX*^{R} ewes, after immunostaining with anti-AMHR2 (12G4) mouse monoclonal antibody. Immunohistochemistry experiment was performed simultaneously on ovarian sections from +/+ and mutant ewes. The panel (E) illustrates AMHR2 immunostaining in antral follicles of 4 to 6 mm in diameter present in +/+ (top) and +/*FecX*^{L}(bottom) Lacaune ovaries. The panel (F) illustrates AMHR2 immunostaining in antral follicles of 3 mm in diameter present in +/+ (top) and +/*FecX*^{R} (bottom) Rasa Aragonesa ovaries. A= Antrum; G = Granulosa cells; O = Oocyte; T = Theca. Scale bar, 100 μm.

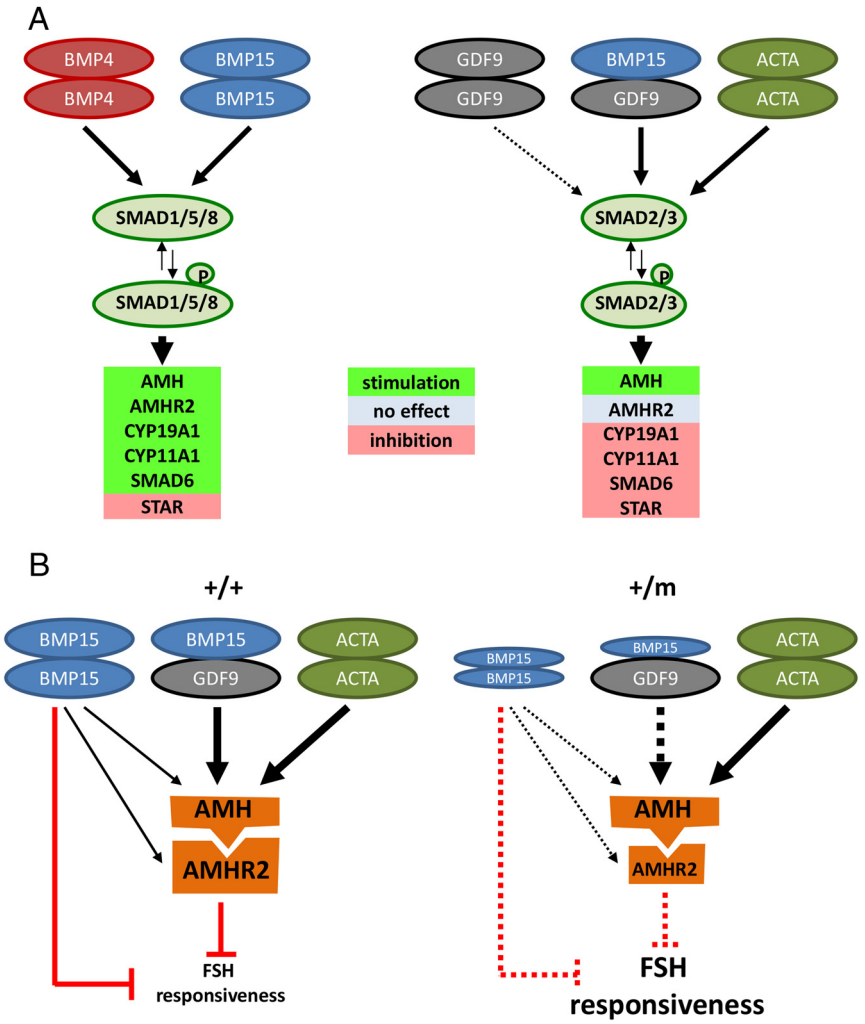


Figure 5. Diagrams illustrating the mechanisms of action of BMP15 and GDF9 on GCs. A, Interactions between BMP15 and GDF9 to regulate their target genes through the phosphorylation of SMAD proteins. BMP15 and GDF9 can act as homodimers or highly potent BMP15/GDF9 heterodimers in granulosa cells (32), with different impacts on the activation of SMAD1/5/8 and SMAD2/3 pathways and the expression of target genes. B, Working hypothesis of regulation of folliculogenesis and ovulation rate by BMP15 through AMH and AMHR2. It is suggested that low BMP15 and low AMH signaling in follicles of ewes carrying a loss-of-function mutation in *BMP15* at the heterozygote state (+/m) both contribute to sensitizing granulosa cells to FSH, thereby enhancing the number of ovulations at each ovarian cycle.

of-function mutations in *BMP15*, had reduced *AMHR2* mRNA levels compared to wild-type mono-ovulating animals, but these mutations had no effect on *AMH* expression. Altogether, these results indicate that BMP15 up-regulates both *AMHR2* and *AMH* expression in GCs, but do so by different mechanisms (Figure 5A). Remarkably, BMP15 is efficient on GCs at different stages of their differentiation and in different species. It is suggested that hyperprolificacy in ewes could result from a decrease in both BMP and AMH inhibitory actions on GCs during the FSH-dependent phase of terminal follicular development (Figure 5B).

From our results, the effects of BMP15 and BMP4 are markedly different from those of GDF9 and Activin A. This is true for *AMHR2* but also for the other BMP/Activin target genes we studied. Indeed, in hGCs, BMP15 stimulates whereas GDF9 inhibits *CYP19A1* expression, and in oGCs, BMP4 and BMP15 stimulate *CYP11A1* and *SMAD6* expression whereas these genes are down-regulated by GDF9 and Activin A respectively. These results are in keeping with a multifactorial regulation of genes involved in folliculogenesis. However, the similarity of BMP4 and BMP15 effects on one hand, and of GDF9 and Activin A effects on the other hand, could be partly due to the fact that they activate the same SMAD1/5/8 and SMAD2/3 pathways respectively. The fact that in oGCs, Activin A mimics the effect of the BMP factors in up-regulating *AMH* mRNA levels, suggests that both SMAD1/5/8 and SMAD2/3 signaling pathways can enhance *AMH* expression in oGCs.

The combination of BMP15 and GDF9 has the same effect than BMP15 on *AMHR2* expression, confirming that GDF9 is not involved in the regulation of this gene (Figure 1 and 2). In contrast, BMP15 and GDF9 exert an additive effect on *SMAD6* and *CYP11A1* expression and a synergistic effect on *STAR* and *CYP19A1* expression. Moreover, a synergistic effect of BMP15 and GDF9 is also noticeable on *AMH* expression in oGCs. As shown by Peng et al (32) for genes involved in cumulus expansion, additive or cooperative effects between these two factors can be explained by the formation of GDF9/BMP15 heterodimers which are more potent than each homodimers alone. The lack, in BMP15 and GDF9, of the seventh cysteine of the Cys-knot domain (which is present in all other members of the TGFß family (33)), likely allows them to dimerize noncovalently with each other. In agreement with this explanation, Mottershead et al (33) demonstrated recently that the potent synergistic actions of GDF9 and BMP15 on GCs does not occur when a covalent form of BMP15, instead of a noncovalent one, is added with GDF9 in the culture medium. Based on these data, we can hypothesize that homodimers of BMP15 are likely necessary to regulate *AMHR2* expression whereas

both homodimers of BMP15 and heterodimers with GDF9 might modulate *AMH* mRNA levels (Figure 5A).

In vivo, BMP15 regulates also differently *AMH* and *AMHR2* expression. Indeed, *AMHR2* but not *AMH* expression is reduced in the GCs from Lacaune and Rasa Aragonesa ewes (Figure 4), which carry a loss-of-function BMP15 mutation at heterozygous state, and which thus lack one "dose" of BMP15. This means that "two doses" of BMP15 are necessary for *AMHR2* expression whereas "one dose" is sufficient for that of *AMH*. Because BMP15 and BMP4 have a similar stimulatory effect on *AMHR2* expression in vitro, the decrease in *AMHR2* expression in GCs from mutant ewes suggests that the factors up-regulating *AMHR2* mRNA, including BMP4, cannot compensate the lack of effect of BMP15. In contrast, the stimulatory effect of Activin A or other factors on *AMH* expression could compensate the loss of one copy of BMP15. Moreover, and as said above, our in vitro results (Figure 2) suggest that the expression of *AMH* could also be supported by the action of BMP15/GDF9 heterodimers with a highly potent stimulatory effect.

The ability of BMP15 and BMP4 to enhance the human *AMHR2* reporter gene activity in ovine GCs (Figure 3) suggests that they could act on the transcription of this gene. BMP15 and BMP4 mainly phosphorylate the SMAD1/5/8 proteins which in turn interact with SMAD4 and translocate to the nucleus. SMAD4 directly binds to SBE (35) but SMAD1/5/8 only weakly bind to this element and prefer GC-rich elements, often referred to BRE (31). The presence of several potential SBE and BRE within the human *AMHR2* promoter (Figure 3C) is in keeping with a direct effect of BMP15 and BMP4 on its transcription through SMAD factors. In addition, other transcription factors, such as SOX9, SF1 and GATA4 present in the *AMHR2* promoter, could act in synergy with SMAD proteins. Supporting this hypothesis, *AMHR2* transcription is up-regulated by SF1 and SOX9 in heterologous cells systems (36). In addition, we have recently found that SF1 binding sites are necessary for BMP4 activation through SMAD1 of the *AMH* promoter in ovine GCs (20).

Both BMPs and AMH are known to repress FSH action in GCs. Indeed in mice, the knock-out of the *AMH* gene leads to more growing follicles, a higher sensitivity to FSH treatment and an earlier depletion of the follicle pool (2). In human, mutations in the *BMP15* gene (37) and in the *AMH* gene (38) at the heterozygous state have been associated with premature ovarian failure or insufficiency. As suggested by Inagaki et al (39), this could be due to impaired levels or decreased activity of mutant proteins, leading to a short period of enhanced fertility followed by a rapid exhaustion of the ovarian reserve. In sheep, as proposed by Fabre et al (40), loss-of-function mutations of BMPs or their receptors at the heterozygous state are likely responsible for the increase in ovulation rate, the reduced inhibiting action of BMP on FSH responsiveness promoting the selection and maintenance of follicles during the follicular phase, when circulating concentration of FSH is decreasing. The stimulatory effect of BMP15 on *AMH* and *AMHR2* expression we have demonstrated in the present study suggests that hyperprolificacy in heterozygous ewes carrying a loss-of-function BMP15 mutation could be due to the combined decrease of BMP15 and AMH inhibitory actions on FSH-dependent folliculogenesis (Figure 5B).

In conclusion, in this study, we have shown that BMP15 stimulates *AMHR2* expression in GCs at different stages of differentiation both in vitro and in vivo. The ability of BMP15 to enhance the activity of a human *AMHR2* reporter gene suggests that it could act on its transcription, but further experiments are required to define its precise mechanism of action. The fact that BMP15 and BMP4 stimulate both *AMH* and *AMHR2* expression suggests an important role of factors of the BMP family, of somatic or oocyte origin, in triggering *AMH* and *AMHR2* expression in primary follicles and/or in sustaining their expression in antral ones, thus emphasizing their inhibitory effect on FSH action on GCs.

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References

1. **Josso N, Picard JY, Rey R, di Clemente N.** Testicular anti-Müllerian hormone : history, genetics, regulation and clinical applications. *Pediatr Endocrinol Rev*. 2006;3(4):347–358.

2. **Visser JA, de Jong FH, Laven JSE, Themmen APN.** Anti-Müllerian hormone: a new marker for ovarian function. *Reproduction*. 2006; 131(1):1–9.
3. **Rey R, Sabourin JC, Venara M et al.** Anti-Müllerian hormone is a specific marker of Sertoli and granulosa-cell origin in gonadal tumors. *Human Pathology*. 2000;31:1202–1208.
4. **La Marca A, Sighinolfi G, Radi D et al.** Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update*. 2010;16(2):113–130.
5. **Bézard J, Vigier B, Tran D, Mauléon P, Josso N.** Immunocytochemical study of anti-Müllerian hormone in sheep ovarian follicles during fetal and postnatal development. *J Reprod Fertil*. 1987;80:509–516.
6. **Baarends WM, Uilenbroek JTJ, Kramer P et al.** Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology*. 1995;136(11):4951–4962.
7. **di Clemente N, Wilson CA, Faure E et al.** Cloning, expression and alternative splicing of the receptor for anti- Müllerian hormone. *Mol Endocrinol*. 1994;8:1006–1020.
8. **Grondahl ML, Nielsen ME, Dal Canto MB et al.** Anti-Mullerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis. *Reprod Biomed Online*. 2011; 22(4):389–398.
9. **McGrath SA, Esquela AF, Lee SJ.** Oocyte-specific expression of growth differentiation factor-9. *Mol Endocrinol*. 1995;9(1):131–136.
10. **Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM.** The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Mol Endocrinol*. 1998;12(12):1809–1817.
11. **Otsuka F, McTavish KJ, Shimasaki S.** Integral role of GDF-9 and BMP-15 in ovarian function. *Mol Reprod Dev*. 2011;78(1):9–21.
12. **Juengel JL, McNatty KP.** The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum Reprod Update*. 2005;11(2):143–160.
13. **Persani L, Rossetti R, Di PE, Cacciatore C, Fabre S.** The fundamental role of bone morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. *Hum Reprod Update*. 2014;20(6):869–883.
14. **Gilchrist RB, Lane M, Thompson JG.** Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update*. 2008;14(2):159–177.
15. **Knight PG, Glister C.** TGF-beta superfamily members and ovarian follicle development. *Reproduction*. 2006;132(2):191–206.
16. **Shimasaki S, Zachow RJ, Li DM et al.** A functional bone morphogenetic protein system in the ovary. *Proc Natl Acad Sci USA*. 1999; 96(13):7282–7287.
17. **Pierre A, Pisselet C, Dupont J et al.** Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells. *J Mol Endocrinol*. 2004;33(3):805–817.
18. **Moore RK, Otsuka F, Shimasaki S.** Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *J Biol Chem*. 2003;278(1):304–310.
19. **Mazerbourg S, Klein C, Roh J et al.** Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol Endocrinol*. 2004;18(3):653–665.
20. **Estienne A, Pierre A, di Clemente N et al.** Anti-Mullerian hormone regulation by the bone morphogenetic proteins in the sheep ovary: deciphering a direct regulatory pathway. *Endocrinology*. 2015; 156(1):301–313.
21. **Ocon-Grove OM, Poole DH, Johnson AL.** Bone morphogenetic protein 6 promotes FSH receptor and anti-Mullerian hormone mRNA expression in granulosa cells from hen prehierarchal follicles. *Reproduction*. 2012;143(6):825–833.
22. **Rico C, Médigue C, Fabre S et al.** Regulation of anti-Mullerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular, and granulosa cell levels. *Biol Reprod*. 2011; 84(3):560–571.
23. **Shi J, Yoshino O, Osuga Y et al.** Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin beta subunits, and anti-Mullerian hormone in human granulosa cells. *Fertil Steril*. 2009;92(5):1794–1798.
24. **Ogura-Nose S, Yoshino O, Osuga Y et al.** Anti-Mullerian hormone (AMH) is induced by bone morphogenetic protein (BMP) cytokines in human granulosa cells. *Eur J Obstet Gynecol Reprod Biol*. 2012; 164(1):44–47.
25. **Martinez-Royo A, Jurado JJ, Smulders JP et al.** A deletion in the bone morphogenetic protein 15 gene causes sterility and increased prolificacy in Rasa Aragonesa sheep. *Anim Genet*. 2008;39(3):294–297.
26. **Bodin L, Di Pasquale E, Fabre S et al.** A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology*. 2007;148(1):393–400.
27. **Taieb J, Grynberg M, Pierre A et al.** FSH and its second messenger cAMP stimulate the transcription of human anti-Mullerian hormone in cultured granulosa cells. *Mol Endocrinol*. 2011;25(4):645–655.
28. **Monniaux D, di Clemente N, Touzé JL et al.** Intrafollicular steroids and anti-mullerian hormone during normal and cystic ovarian follicular development in the cow. *Biol Reprod*. 2008;79(2):387–396.
29. **Salhi I, Cambon-Roques S, Lamarre I et al.** The anti-Mullerian hormone type II receptor: insights into the binding domains recognized by a monoclonal antibody and the natural ligand. *Biochem J*. 2004; 379(Pt 3):785–793.
30. **Massagué J, Hata A, Liu F.** TGF-ß signalling through the Smad pathway. *Trends Cell Biology*. 1997;7:187–192.
31. **LopezRovira T, Chalaux E, Massagué J, Rosa JL, Ventura F.** Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem*. 2002;277(5):3176–3185.
32. **Peng J, Li Q, Wigglesworth K et al.** Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proc Natl Acad Sci U S A*. 2013;110(8): E776–E785.
33. **Mottershead DG, Sugimura S, Al-Musawi SL et al.** Cumulin, an Oocyte-secreted Heterodimer of the Transforming Growth Factor-beta Family, Is a Potent Activator of Granulosa Cells and Improves Oocyte Quality. *J Biol Chem*. 2015;290(39):24007–24020.
34. **Shimasaki S, Moore RK, Otsuka F, Erickson GF.** The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev*. 2004;25(1):72–101.
35. **Lopez-Rovira T, Chalaux E, Massagué J, Rosa JL, Ventura F.** Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem*. 2002;277(5):3176–3185.
36. **Santa Barbara Pd, Moniot B, Poulat F, Boizet B, Berta P.** Steroidogenic factor-1 regulates the transcription of the human anti- Müllerian hormone receptor. *J Biol Chem*. 1998;273:29654–29660.
37. **Di Pasquale E, BeckPeccoz P, Persani L.** Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Amer J Hum Genet*. 2004; 75(1):106–111.
38. **Alvaro MB, Imbert R, Demeestere I et al.** AMH mutations with reduced in vitro bioactivity are related to premature ovarian insufficiency. *Hum Reprod*. 2015;30(5):1196–1202.
39. **Inagaki K, Shimasaki S.** Impaired production of BMP-15 and GDF-9 mature proteins derived from proproteins WITH mutations in the proregion. *Mol Cell Endocrinol*. 2010;328(1–2):1–7.
40. **Fabre S, Pierre A, Mulsant P et al.** Regulation of ovulation rate in mammals: contribution of sheep genetic models. *Reprod Biol Endocrinol*. 2006;4:20.