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Ewes from the Booroola strain of Australian Mérino sheep are characterized by high ovulation rate and litter size. This phenotype is due to the action of the *FecB^B* allele of a major gene named *FecB*, as determined by statistical analysis of phenotypic data. By genetic analysis of 31 informative half-sib families from heterozygous sires, we showed that the *FecB* locus is situated in the region of ovine chromosome 6 corresponding to the human chromosome 4q22–23 that contains the bone morphogenetic protein receptor IB (*BMPR-IB*) gene encoding a member of the transforming growth factor-β (TGF-β) receptor family. A nonconservative substitution (Q249R) in the *BMPR-IB* coding sequence was found to be associated fully with the hyperprolificacy phenotype of Booroola ewes. *In vitro*, ovarian granulosa cells from *FecB^B*/*FecB^B* ewes were less responsive than granulosa cells from *FecB$^+$*/*FecB$^+$* ewes to the inhibitory effect on steroidogenesis of GDF-5 and BMP-4, natural ligands of BMPR-IB. It is suggested that in *FecB^B*/*FecB^B* ewes, BMPR-IB would be inactivated partially, leading to an advanced differentiation of granulosa cells and an advanced maturation of ovulatory follicles.

In the Australian Booroola Mérino sheep, the hyperprolificacy is because of the action of a single autosomal gene (*FecB*) that influences the number of ovulations per estrous cycle in the ewe. Ewes that are homozygous *FecB^B*/*FecB^B*, heterozygous *FecB^B*/*FecB$^+$*, and noncarriers *FecB$^+$*/*FecB$^+$* of the *FecB^B* Booroola mutation can be segregated on the basis of ovulation rate recordings of five or more, three or four, and one or two, respectively (1, 2). Extensive physiological analysis has established that the product of the *FecB* gene influences both pituitary and ovarian activity. Particularly in ovarian follicles, this gene product acts directly or indirectly by inducing a precocious maturation of ovarian follicles, which ovulate at a smaller size (3). The identity of the *FecB* gene and the physiological mechanisms leading to the differences in ovulation rate remain unknown (4).

With the aim to identify the *FecB* gene, we established a detailed genetic map of the ovine *FecB* region. On sheep chromosome 6, the *FecB* gene is located between the *SPP1* and *EGF* genes (5, 6). As extensive homology has been demonstrated with the orthologous 4q21–25 region of human chromosome 4 (7), we developed a comparative mapping strategy that converged on a chromosomal region containing the bone morphogenetic protein receptor IB (*BMPR-IB*) gene. In this paper we show that a mutation in the coding sequence of the *BMPR-IB* gene was found to be associated fully with the *FecB^B* Booroola mutation and that ovarian cells from *FecB^B*/*FecB^B* ewes are less responsive to ligands of the BMPR-IB receptor than ovarian cells from *FecB$^+$*/*FecB$^+$* ewes.

Materials and Methods

Animals. Half-sib families were constituted by backcrossing 31 heterozygous *FecB^B*/*FecB$^+$* sires to Mérinos d'Arles dams. Classification of the progeny ewes was carried out by maximum-likelihood analysis as described (8). The threshold was fixed at 0.85, and 400 ewes could be classified as being *FecB^B*/*FecB$^+$* or *FecB$^+$*/*FecB$^+$*. Dams were presumed to be *FecB$^+$*/*FecB$^+$*. DNA from blood cells was extracted as described (9).

For *in vitro* culture experiments, cyclic *FecB^B*/*FecB^B* ($n = 12$) and *FecB$^+$*/*FecB$^+$* ($n = 12$) Mérinos d'Arles ewes were treated with intravaginal progestagen sponges (fluorogestone acetate, 40 mg, Intervet, Angers, France) for 13 days to synchronize estrus. Ovaries from both genotypes were recovered after ovariectomy in the late follicular phase 36 h after sponge removal. All procedures were approved by the agricultural and scientific research government committees in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

Bacterial Artificial Chromosomes (BACs) and Yeast Artificial Chromosomes (YACs). Five ruminant large insert libraries were used: three BAC libraries [sheep (10), goat (11), and cattle] and two bovine YAC libraries. Pulse-field gel electrophoresis analysis was carried out on a CHEF apparatus (Amersham Pharmacia) on 1 μg of *Not*I-digested BAC DNA. BAC end-sequencing was carried out for 99 elongation cycles on 1 μg BAC DNA with M13 and M13rev 24-mer vector primers by using ABI dRhodamine Terminator Cycle Sequencing Ready Reaction kits from Applied Biosystems.

Marker Analysis. PCRs, single-strand conformational polymorphism, and microsatellite analyses were conducted following

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Abbreviations: BMPR-IB, bone morphogenetic protein receptor IB; FSH, follicle-stimulating hormone; TGF, transforming growth factor; BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; RT, reverse transcription.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF298885).

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Table 1. Primers and PCR amplification conditions

Name	Type*	Alleles†	Species	Primer pairs		PCR‡
				Markers		
BMS2508	ms	3	Cattle	AGGTTGACTTCTGTGTCTTTTC	GTTTCTTAGGGGAGTGTTGATT	56/2.5
ETL1	snp	2	Sheep	CATCAATGGGCCTGTGAAAT	CAAACAATCACCAACTAATCC	54/3
471U	ms	3	Sheep	CTACAATAAATAATGAGGTGAAA	TGAGTAGAGACAAAGCTATAAA	52/3
UNC5C (GC101)	ms	7	Sheep	ATCCTCACCCTTCAAACAG	CTGGGGAGTTTTCTCTGAC	56/1.5
UNC5C (GC102)	snp	3	Sheep	TTCCCTCTTGCTGTATCTTCT	AAACACTGGGTGACTGACTC	58/1.5
300R	snp	3	Sheep	CCAAACATCAGCAAACCATAA	CTGTGACCATCTCTCTGTG	56/3
320R	snp	4	Sheep	TCACCTTGAATCATTTCTTTAG	GTGGTGTTTGGAACTTTGTAA	55/1.5
300U	ms	12	Sheep	GGGGGTTCCTTGTAGGTTTGTG	GGAAGTGCAGAGAGTCCCATAC	66/3
Bulge5	ms	6	Cattle	CACAAACATTCGGACATGACC	TGAGGAGGGCACATCCATTG	54/3
LSCV043	ms	6	Goat	CCAGAATATAGAGTTTTGTCAAG	GCCTGATTTGTATTTGTATGAG	53/3
				BMPR-IB analysis		
OB1	snp	4	Sheep	CAGAACGGAATGAATGTAATAA	AGAACAGGGTAAGGAAGTAAA	55/3
OB2	snp	2	Sheep	AGATTGGAAAAGGTCGCTATG	ACCCTGAACATCGCTAATACA	56/1.5
B3	cDNA		Human	TGACAAGAGAGGAAACAAAAAG	AGGACCAAGAGCAAACTACA	55/2
B4	cDNA		Human/sheep	GACACTCCCATTCCTCATCA	GCAGTTTCTCCCGCCTACAG	55/2

*ms, microsatellite; snp, single nucleotide polymorphism; cDNA, primers for cDNA amplification.
†Number of alleles in the sheep backcross population.
‡Annealing temperature (°C) and magnesium concentration (mM).

standard procedures by using markers described in Table 1. Microsatellite *300U* was analyzed by autoradiography of primer-labeled PCR fragments. Microsatellite *LSCV043* was analyzed on Metaphor gels. All markers were sequenced. Genetic segregation analysis was done with the CRIMAP software (12) using the ''build'' and ''flips'' options. The FecB genotype of 12 progeny individuals was inconsistent with their genotype at all marker loci. These animals were considered to correspond to misclassification cases and were excluded from the analysis.

BMPR-IB Analysis. Extraction of mRNA from granulosa cells was realized as described by Chomczynski and Sacchi (13). cDNA synthesis was achieved by RT Superscript II from GIBCO/BRL with a $(dT)_{15–18}$ primer. Coding sequences were amplified with primer pairs B3 and B4 (Table 1). Sequences were controlled with different internal primers. Determination of BMPR-IB intron-exon junctions was carried out by sequencing subclones of BACs 118b4 and 382c4 with internal BMPR-IB primers. The Q249R mutation was tested by single-strand conformation analysis using primers flanking the A → G transition (OB2 in Table 1). Reverse transcription (RT)-PCR was performed by using primers 5′-CGAATGAAGTTGACATACCAC-3′ and 5′-TCGTAAGAGGGGTCACTGG-3′ located in two different exons at positions 1,129 and 1,342 to discriminate eventual amplification from genomic DNA contaminants. We also designed primers 5′-CTGACCTGCCGCCTGGAGAAA-3′ and 5′-GTAGAAGAGTGAGTGTCGCTGTT-3′ to assess the expression of ovine G3PDH mRNA (GenBank accession no. U39091), used as an internal control of reverse transcription RT-PCR. Multiple sequence alignments were performed by using MULTALIN software (14).

Granulosa Cell Cultures. Granulosa cells were recovered from antral follicles of 1–3 mm in diameter as described (15). Granulosa cells were seeded at 100,000 viable cells per well in 96-well plates and cultured for 96 h at 37°C with 5% CO_2 in serum-free McCoy's 5a medium (Sigma) containing insulin (100 ng/ml, Sigma) and insulin-like growth factor-1 (10 ng/ml, CIBA–Geigy) according to a previously described method (16). Cultures were performed with or without follicle-stimulating hormone (FSH, 5 ng/ml, purified ovine FSH-20, lot n°AFP-7028D, National Hormone Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases) and in the presence of various concentrations (0, 1, 5, 10, and 50 ng/ml) of dimeric recombinant human GDF-5 (Biopharm, Heidelberg, Germany), recombinant human BMP-4 (Genetics Institute, Cambridge, MA), or 50 ng/ml of monomeric recombinant human GDF-5 (Biopharm) as negative control. Each combination of treatments was tested in triplicate for each culture and in three independent cultures. In four independent cultures, proliferation was assessed by studying [^{3}H]thymidine [0.25 μCi/ml (1 Ci = 37 GBq), DuPont] incorporation for 2 h after 48 h of culture, and the labeling index (percentage of [^{3}H]thymidine-labeled cells) was determined as described (17).

Progesterone RIA. Progesterone concentrations in the culture media were measured in a direct RIA as described (18). The limit detection of the assay was 12 pg per tube, and the intra-assay coefficient of variation was 10%.

Molecular Modeling. Modeling of BMPR-IB was performed with the SWISSMODEL application (http://www.expasy.ch/swissmod/SWISS-MODEL.html) by using the human TβR-I/FKBP12 complex as template (Protein Data Bank identifier 1B6CB). Structure visualization was performed by using the INSIGHT II program package (Molecular Simulations, Waltham, MA).

Data Analysis. All experimental data are presented as mean ± SEM. For results of progesterone secretion, data were expressed as percentages of the amount of progesterone secreted by cells cultured in the absence of GDF-5 or BMP-4. For each genotype and culture condition (with or without FSH), the effects of ligands was studied by two-way ANOVA to appreciate the action of each dose of ligand as well as the variation between independent cultures. For each dose of ligand, the effect of genotype was studied by one-way ANOVA. All differences with $P > 0.05$ were considered nonsignificant.

Results

Genetic Analysis of the *FecB* Region. Five ruminant large insert (BAC or YAC) libraries were screened with (*i*) microsatellites of bovine chromosome 6 and (*ii*) comparative anchored tagged sequences corresponding to genes located on human chromosome 4q21–24, between the prepromultimerin and microsomal triglyceride transfer protein genes. Genetic markers were developed by partial sequencing of the isolated BACs or YACs, either

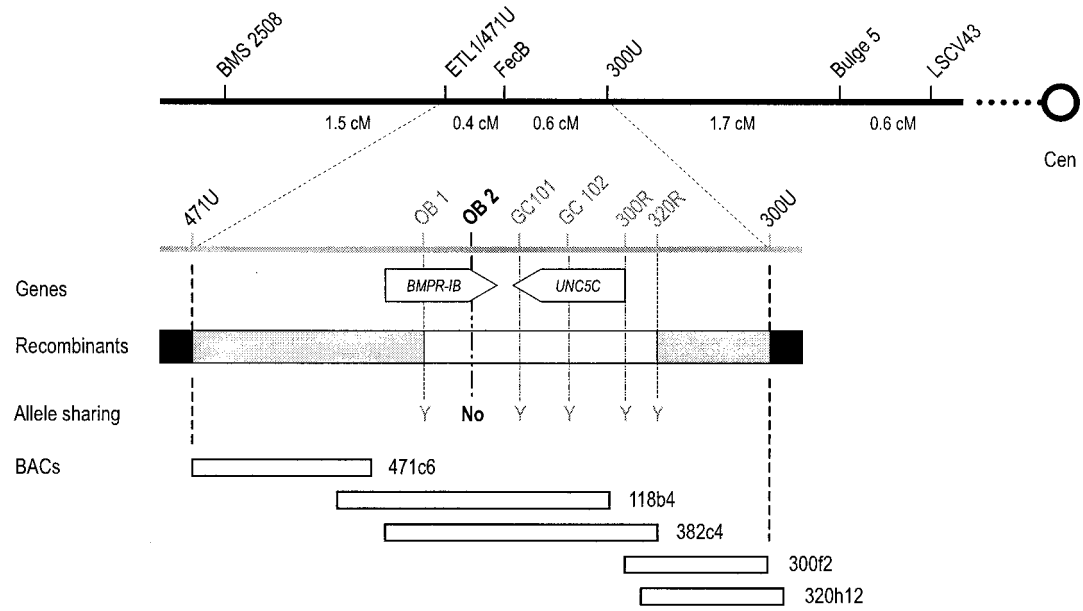

Fig. 1. Physical and genetic map of the *FecB* region on ovine chromosome 6. The *FecB* locus is flanked by the two closest recombinant markers, *471U* and *300U*. No recombination was observed between FecB and markers *OB1*, *OB2*, *GC101*, *GC102*, *300R*, and *320R*. Arrows correspond to the orientation of *BMPR-IB* and *UNC5C* coding sequences. The 5′ part of the *UNC5C* coding sequence was not present in the BAC 382c4. Recombinants: white box, zero-recombinant zone; gray boxes, zone with zero or one recombinant; black boxes, at least one recombinant with FecB. Allele sharing between wild-type and carrier animals for the $FecB^B$ allele is indicated: Y, yes; N, no; open boxes, BACs; Cen, centromere; *ETL1*, enhancer trap locus 1.

in the form of microsatellites or single nucleotide polymorphisms (Table 1). Linkage between markers including *FecB* was tested on a backcross experimental population of 31 half-sib families from heterozygous $FecB^B/FecB^+$ sires (400 informative meiosis for *FecB*). *FecB* was found to be situated between *ETL1* (<1 centimorgan) and the microsatellite *Bulge5*, isolated from a bovine YAC screened with *PDHA2*-specific primers (Fig. 1). The interval between *ETL1* (position 477 centirays) and *PDHA2* (483.7 centirays) on the GB4 human irradiation map (http://www.ncbi.nlm.nih.gov/genemap/) contains two identified genes at position 480 centirays: *BMPR-IB* and *UNC5C*. Both genes were found in the same sheep BAC clones, 118b4 and 382c4. Two markers in the ends of the adjacent BACs, 471c6 and 300f2, defined critical recombination events on both sides of *FecB*. No recombination was observed between *FecB* and any marker from the 382c4 BAC clone (Fig. 1).

***BMPR-IB* Sequence Analysis.** The BMP system has been shown recently to be expressed in rat ovaries and to affect steroid synthesis of granulosa cells (19). The *BMPR-IB* gene thus was considered as a positional candidate for *FecB* and studied in more detail. To determine the cDNA sequence of ovine *BMPR-IB*, primer pairs based on the human and mouse sequences were used to amplify a coding sequence of 1,509 bp from both $FecB^+/FecB^+$ and $FecB^B/FecB^B$ granulosa cells, as well as 47 and 211 bp of 5′ and 3′ untranslated sequences, respectively. The nucleotide sequence of the wild-type allele showed 93% identity with the human *BMPR-IB* coding sequence. The predicted protein sequence (Fig. 2*a*) showed 98% identity with human BMPR-IB over a 502-aa overlap. The ovine coding sequence was contained in the overlap region between BACs 118b4 and 382c4 and was found to be divided into 10 exons. BLAST comparison of ovine BAC 118b4 sequences with the orthologous human BAC 115M7 (GenBank accession no. AC009920) showed a total conservation of the exons-introns organization of the gene between sheep and human.

The nucleotide sequence obtained from the $FecB^B$ allele was identical to the wild-type allele, except for a A → G transition at position 746, substituting the glutamine present in the wild-type sequence as well as in human and mouse sequences, with an arginine (CAG → CGG, Q249R, Fig. 2*b*). Three additional polymorphisms were observed in wild-type sequences: a conservative substitution at position 1,113 of the coding sequence and two substitutions in the 3′ untranslated region. The backcross population and 120 additional animals from 12 different breeds were genotyped by single-strand conformation analysis by using primers flanking the A → G transition. Only two alleles were obtained, which were found to be in total linkage disequilibrium with *FecB*. Indeed, the 249R allele was found in all $FecB^B$ carrier animals but in no control animal from Mérinos d'Arles ($n = 190$) or other breeds ($n = 120$), whereas allele sharing was observed for all the other markers from the nonrecombinant region. This is consistent with the full association of the 249R mutation in BMPR-IB with the Booroola phenotype.

Effect of BMPR-IB Ligands on Ovarian Granulosa Cells *in Vitro*. To investigate a physiological role of BMPR-IB in ovine granulosa cells, we have studied the effects of recombinant GDF-5 or BMP-4, both ligands known to exert their actions specifically through type I BMP receptors (20, 21), on proliferation and secretion of progesterone by granulosa cells from $FecB^+/FecB^+$ and $FecB^B/FecB^B$ ewes *in vitro*. In basal conditions, $FecB^+/FecB^+$ granulosa cells exhibited a higher ^{3}H-thymidine-labeling index than $FecB^B/FecB^B$ cells ($P < 0.001$), confirming that the presence of the $FecB^B$ mutation is associated with a decrease in proliferative activity of granulosa cells in small antral follicles (17, 22). No effect of GDF-5 was observed on labeling index, but a slight increase was detected in the presence of BMP-4 (50 ng/ml) for $FecB^+/FecB^+$ cells (12.15 ± 1.32% vs. 7.55 ± 0.49%, BMP-4 vs. control, $n = 4$, $P < 0.05$), that did not occur for $FecB^B/FecB^B$ cells (6.45 ± 1.32% vs. 4.65 ± 1.06%, BMP-4 vs. control, $n = 4$, nonsignificant).

As previously reported for small antral follicles (17), progesterone secretion by granulosa cells slightly increased in both

Fig. 2. Predicted amino acid sequence of ovine *BMPR-IB* cDNA. (*a*) The mutation site between wild type and $FecB^B/FecB^B$ is shown with a stippled box. Also indicated are the transmembrane domain (open box), GS domain (underlined), the limits of the kinase domain (bent arrows), the ATP binding region signature (under brackets), and the L45 loop (in bold). (*b*) Comparison of partial amino acid sequences of different members of type I TGF-β receptor family from different species. Numbers indicate amino acid positions in the ovine BMPR-IB protein.

genotypes in the presence of 5 ng/ml FSH (1.67 ± 0.12- and 2.17 ± 0.42-fold increase for $FecB^+/FecB^+$ and $FecB^B/FecB^B$, respectively). When added to the culture medium, GDF-5 and BMP-4 inhibited progesterone secretion by granulosa cells dose-dependently, in the absence and presence of FSH and in both genotypes (Fig. 3). Interestingly, granulosa cells from $FecB^B/FecB^B$ ewes were less responsive than those from $FecB^+/FecB^+$ to GDF-5 and BMP-4 action, regardless of the presence or absence of FSH. Indeed, the first dose of GDF-5 that was effective in inhibiting progesterone secretion was 5 ng/ml for $FecB^+/FecB^+$ cells ($P < 0.001$) compared with 10 ng/ml for $FecB^B/FecB^B$ cells ($P < 0.01$). Moreover, the inhibition of progesterone secretion was significantly stronger for $FecB^+/FecB^+$ cells than for $FecB^B/FecB^B$ cells in the presence of 50 ng/ml of GDF-5 ($P < 0.001$) or in the presence of BMP-4 from 5 ng/ml onward ($P < 0.001$). The lower responsiveness of $FecB^B/FecB^B$ granulosa cells could not be explained by a lower expression of BMPR-IB mRNAs as indicated by RT-PCR experiments (Fig. 4). More likely, these results suggest a difference of functionality of the BMPR-IB receptor between $FecB^+/FecB^+$ and $FecB^B/FecB^B$ granulosa cells.

Molecular Modeling of the BMPR-IB Mutation. Recently, the crystal structure of human type I transforming growth factor (TGF)-β receptor (TβR-I) in complex with FKBP12, known as a negative modulator of TβR-I family member activity, has been solved (23). Modeling of ovine BMPR-IB using the TβR-I/FKBP12 complex as template indicated a very similar structure with no insertion or deletion supported by a high level of sequence identity (68%). The Q249 residue in ovine BMPR-IB lies critically between the GS domain and the L45 loop (Fig. 2*a*). In the human TβR-I structure, Q250, which corresponds to the BMPR-IB Q249, is located at the C terminus of the αC helix that makes contacts with the FKBP12 molecule (Fig. 5*A*). Q250 does not form any obvious bond with the GS region nor with FKBP12 (Fig. 5*B*). In contrast, the Q250R mutation is predicted to form a strong hydrogen bond with the main-chain oxygen of FKBP12 P88 (Fig. 5*C*, dashed line). This interaction between FKBP12 and the receptor should be reinforced by a nearly parallel

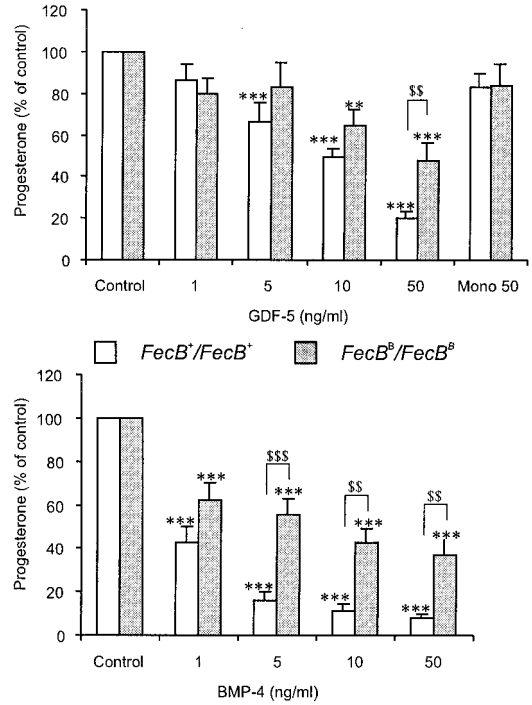

Fig. 3. Effects of GDF-5 (*Upper*) and BMP-4 (*Lower*) on progesterone secretion by $FecB^B/FecB^B$ and $FecB^+/FecB^+$ granulosa cells *in vitro*. Granulosa cells from $FecB^B/FecB^B$ and $FecB^+/FecB^+$ antral follicles of 1–3 mm in diameter were cultured for 96 h in serum-free conditions with 10 ng/ml insulin-like growth factor-1. Cultures were performed in the presence or absence (Control) of different concentrations of BMP-4, dimeric GDF-5, or 50 ng/ml monomeric GDF-5 as negative control (Mono 50) and with or without FSH (5 ng/ml) in culture medium. Results represent progesterone secretion by granulosa cells between 48 and 96 h in three independent cultures. Data are expressed as percentages of the amount of progesterone secreted by cells cultured in the absence of GDF-5 or BMP-4. *In vitro*, FSH had no significant effect on the inhibition rate induced by both ligands, and data include FSH-treated and untreated cells. **, $P < 0.01$ and ***, $P < 0.001$, BMP-4 or GDF-5 vs. control; $$, $P < 0.01$ and $$$, $P < 0.001$, $FecB^B/FecB^B$ vs. $FecB^+/FecB^+$.

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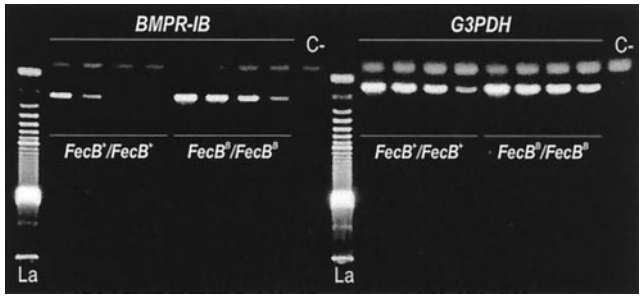

Fig. 4. RT-PCR analysis of *BMPR-IB* from sheep granulosa cells. Successive dilutions of total granulosa cDNA (1 ng and 250, 50, and 10 pg, from left to right for each group) were used as template for PCR amplification of *BMPR-IB* (*Left*) and *G3PDH* (*Right*). L, 100-bp ladder; C, negative control (no cDNA).

stacking interaction between π electrons of R250 and FKBP12 H87 (Fig. 5*C*, arrow). The same prediction can be made for the ovine BMPR-IB model that has been fitted on the TβR-I/FKBP12 complex. Thus, the Q249R mutation may enhance the interaction between FKBP12 and BMPR-IB considerably, leading to a stronger inhibition of receptor activity.

Discussion

We have identified a mutation (Q249R) in the coding sequence of the *BMPR-IB* gene, which seems fully associated with the $FecB^B$ allele and was not found in any Mérinos d'Arles control animal or in 120 additional animals from 12 different breeds. The *BMPR-IB* gene was the only gene situated in the nonrecombinant region expressed in the ovary. Indeed, there was no amplification in RT-PCR experiments on sheep ovarian cDNA by using *UNC5C*-specific primers. In addition, there was no evidence of a regulatory mutation in Booroola animals leading to differential splicing or to a significantly impaired expression of the *BMPR-IB* gene. Indeed, RT-PCR experiments performed with different BMPR-IB primers on mRNA from $FecB^+/FecB^+$ or $FecB^B/FecB^B$ ovarian granulosa cells always yielded a single band and did not indicate a lower expression of BMPR-IB mRNA in $FecB^B/FecB^B$ cells. Finally, in contrast with the complete linkage disequilibrium observed for the Q249R mutation, allele sharing between $FecB^B$- and $FecB^+$-associated haplotypes was observed at all other marker loci in the nonrecombinant region, including *BMPR-IB* sequences.

BMPs exert their biological effects after binding to type I receptor, BMPR-IA or BMPR-IB, which dimerizes with type II receptor, BMPR-II (24–26). Our observations indicate a strong inhibitory effect of GDF-5 and BMP-4, two natural ligands of BMPR-IB, on progesterone secretion by sheep granulosa cells in basal and FSH-stimulated conditions with no clear effect on cell proliferation. Interestingly, Shimasaki *et al.* (19) have reported recently similar effects of BMP-4 and BMP-7 on progesterone production by rat granulosa cells but only in the presence of FSH. Our results clearly indicate that granulosa cells from $FecB^B/FecB^B$ ewes were less responsive than cells from $FecB^+/FecB^+$ ewes to GDF-5 or BMP-4 inhibitory action on progesterone secretion. This difference between genotypes was not explained by a clear difference in BMPR-IB RNA and protein levels as assessed by RT-PCR (present results) and immunohistochemistry, respectively (data not shown), although an effect of the mutation on receptor stability cannot be ruled out completely. Moreover, it could be argued that the decrease in responsiveness of $FecB^B/FecB^B$ cells might be caused by a more advanced differentiation stage of granulosa cells of $FecB^B$ carrier ewes

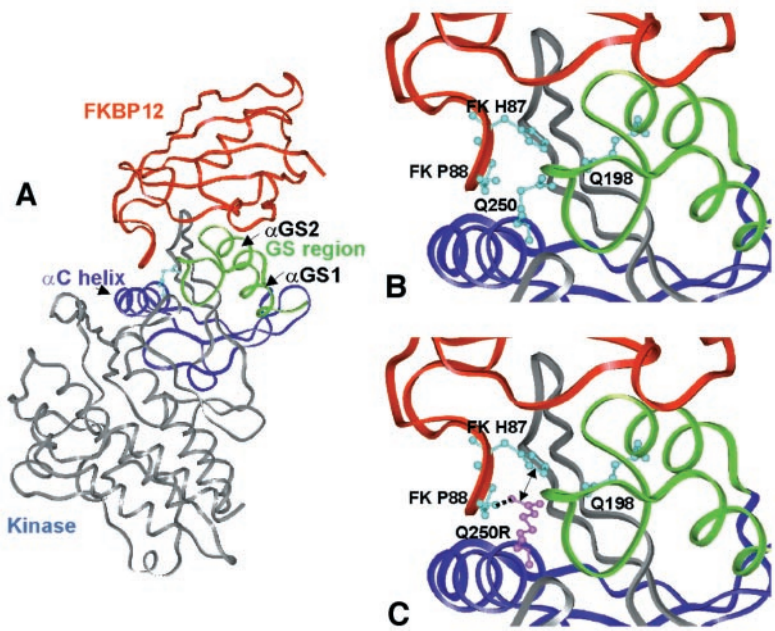

Fig. 5. Modeling of the effects of Q250R substitution on the human TβR-I/FKBP12 complex structure. (*A*) Experimental structure of TβR-I in complex with FKBP12 (Protein Data Bank identifier 1B6C). Q250, located at the C terminus of αC helix, is shown in a ball-and-stick representation. The ovine BMPR-IB model is very similar to TβR-I (0.1 Å rms between the two structures: 326 superimposable Cα). (*B*) Q250 is sandwiched between the FKBP12 flap (one side of the FKBP12 active site in which helix αGS2 is embedded) and the αGS1/αGS2 loop (GS region), which undergoes phosphorylation after kinase activation. The movement of αC helix, allowed by the GS region phosphorylation and/or FKBP12 dissociation, is likely to be responsible partly for kinase activation (23). Q250 does not form any obvious bond with the GS region nor with FKBP12 (Q198 Oε1 atom, the nearest neighbor, is 3.5 Å distant from Q250 Nε2 atom). (*C*) Consequence of the Q250R mutation. In the most probable conformer, R250 is predicted to form a strong hydrogen bond through its Nη1 atom with the main chain oxygen of FKBP12 P88 (dashed line). This interaction between FKBP12 and the receptor should be reinforced by a nearly parallel stacking interaction between π electrons of R250 and FKBP12 H87 (e.g., 3.2 Å between R250 Nε and FKBP12 H87 Nε2; see arrow). The same prediction can be made for the ovine BMPR-IB model that has been fitted on the TβR-I/FKBP12 complex.

compared with noncarriers when isolated from the same follicle size class (17, 27–30). However, in preliminary studies with sheep granulosa cells, similar inhibitory effects of GDF-5 or BMP-4 have been observed on progesterone secretion of fully differentiated cells isolated from preovulatory follicles compared with less mature cells isolated from small antral follicles. Moreover, activin A, another member of the TGF-β family, also inhibited progesterone secretion, but unlike GDF-5 or BMP-4, it acted with the same efficiency on $FecB^B/FecB^B$ and $FecB^+/FecB^+$ granulosa cells from 1- to 3-mm diameter follicles (S.F., C.P., D.M., and P. Mouget, unpublished data). Thus it is suggested strongly that the BMP pathway is altered specifically in $FecB^B/FecB^B$ ovaries. Altogether, these results reinforce the hypothesis that the presence of the Q249R mutation in the BMPRI-B receptor is responsible for an alteration of its activity. This mutation likely leads to a partial rather than a complete inactivation of BMPR-IB. Indeed, in the mouse, targeted invalidation of the *BMPR-IB* gene causes clear skeletal defects (31), whereas no defect in skeletal development of $FecB^B$-carrier sheep has been reported yet. The Q249R mutation is not situated in a region previously shown to be involved in signal transduction (23). However, modelization of the BMPR-IB three-dimensional structure indicates that the Q249R substitution likely reinforces the interaction of BMPR-IB with the immunophilin FKBP12, a negative modulator of type I TGF-β receptor family activity (32, 33). One could hypothesize that the mutation would lead to a stronger inhibition of BMPR-IB activity by FKBP12, resulting in a decrease in cell responsiveness to specific ligands. Nevertheless, the role of FKBP12 in BMPR-IB action is unknown presently. As proposed for TβR1 (34), FKBP12 both might prevent spontaneous transphosphorylation of type I BMP receptors by BMPR-II in the absence of ligand and modulate receptor activity in the presence of ligand.

Overall, we have shown that the Q249R mutation in BMPR-IB is associated fully with the hyperprolific phenotype of Booroola Mérino ewes. In $FecB^B$ carrier ewes, Q249R substitution would impair the inhibitory effect of BMPR-IB on granulosa cell steroidogenesis, leading to their advanced differentiation and an advanced maturation of follicles. Interestingly, very recent findings have shown correlations between hyperprolific phenotypes in Inverdale and Hanna ewes, and mutations in BMP-15, another member of the TGF-β family, which has been shown to be expressed specifically by oocytes (35). However, in a *Xenopus laevis* mesoderm induction assay, BMP-4 but not BMP-15 was shown to induce ventral mesoderm, suggesting that BMP-15 is not a physiological ligand of BMPR-IB (36). Nevertheless, all these results underline the crucial role of BMP family members and their receptors in the regulation of terminal folliculogenesis and the control of ovulation rate.

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1. Davis, G. H., Montgomery, G. W., Allision, A. J., Kelly, R. W. & Bray, A. R. (1982) *N. Z. J. Agric. Res.* **25,** 525–529.
2. Piper, L. R., Bindon, B. M. & Davis, G. H. (1985) in *Genetics of Reproduction in Sheep*, eds. Land, R. B. & Robinson, D. W. (Butterworths, London), pp. 115–125.
3. McNatty, K. P. & Henderson, K. M. (1987) *J. Steroid Biochem.* **27,** 365–373.
4. Montgomery, G. W. (2000) *Rev. Reprod.* **5,** 25–37.
5. Montgomery, G. W., Crawford, A. M., Penty, J. M., Dodds, K. G., Ede, A. J., Henry, H. M., Pierson, C. A., Lord, E. A., Galloway, S. M., Schmack, A. E., *et al.* (1993) *Nat. Genet.* **4,** 410–414.
6. Montgomery, G. W., Lord, E. A., Penty, J. M., Dodds, K. G., Broad, T. E., Cambridge, L., Sunden, S. L., Stone, R. T. & Crawford, A. M. (1994) *Genomics* **22,** 148–153.
7. Lanneluc, I., Mulsant, P., Saidi-Mehtar, N. & Elsen, J. M. (1996) *Cytogenet. Cell Genet.* **72,** 212–214.
8. Lanneluc, I., Drinkwater, R. D., Elsen, J. M., Hetzel, D. J., Nguyen, T. C., Piper, L. R., Thimonier, J., Harrison, B. & Gellin, J. (1994) *Mamm. Genome* **5,** 26–33.
9. Montgomery, G. W. & Size, J. A. (1990) *N. Z. J. Agric. Res.* **33,** 437–441.
10. Vaiman, D., Billault, A., Tabet-Aoul, K., Schibler, L., Vilette, D., Oustry-Vaiman, A., Soravito, C. & Cribiu, E. P. (1999) *Mamm. Genome* **10,** 585–587.
11. Schibler, L., Vaiman, D., Oustry, A., Guinec, N., Dangy-Caye, A. L., Billault, A. & Cribiu, E. P. (1998) *Mamm. Genome* **9,** 119–124.
12. Green, P., Falls, K. & Crocks, S. (1990) CRIMAP Documentation (Washington University School of Medicine, St. Louis).
13. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
14. Corpet, F. (1988) *Nucleic Acids Res.* **16,** 10881–10890.
15. Monniaux, D. & Pisselet, C. (1992) *Biol. Reprod.* **46,** 109–119.
16. Campbell, B. K., Scaramuzzi, R. J. & Webb, R. (1996) *J. Reprod. Fertil.* **106,** 7–16.
17. Monniaux, D., Monget, P., Pisselet, C., Fontaine, J. & Elsen, J. M. (2000) *Biol. Reprod.* **63,** 1205–1213.
18. Saumande, J. (1991) *J. Steroid Biochem. Mol. Biol.* **38,** 189–196.
19. Shimasaki, S., Zachow, R. J., Li, D., Kim, H., Iemura, S., Ueno, N., Sampath, K., Chang, R. J. & Erickson, G. F. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 7282–7287.
20. Nishitoh, H., Ichijo, H., Kimura, M., Matsumoto, T., Makishima, F., Yamaguchi, A., Yamashita, H., Enomoto, S. & Miyazono, K. (1996) *J. Biol. Chem.* **271,** 21345–21352.
21. ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C. H. & Miyazono, K. (1994) *J. Biol. Chem.* **269,** 16985–16988.
22. Driancourt, M. A., Cahill, L. P. & Bindon, B. M. (1985) *J. Reprod. Fertil.* **73,** 93–107.
23. Huse, M., Chen, Y. G., Massague, J. & Kuriyan, J. (1999) *Cell* **96,** 425–436.
24. Liu, F., Ventura, F., Doody, J. & Massague, J. (1995) *Mol. Cell. Biol.* **15,** 3479–3486.
25. Heldin, C. H., Miyazono, K. & ten Dijke, P. (1997) *Nature (London)* **390,** 465–471.
26. Kawabata, M., Imamura, T. & Miyazono, K. (1998) *Cytokine Growth Factor Rev.* **9,** 49–61.
27. Henderson, K. M., Kieboom, L. E., McNatty, K. P., Lun, S. & Heath, D. (1985) *J. Reprod. Fertil.* **75,** 111–120.
28. McNatty, K. P., Lun, S., Heath, D. A., Ball, K., Smith, P., Hudson, N. L., McDiarmid, J., Gibb, M. & Henderson, K. M. (1986) *J. Reprod. Fertil.* **77,** 193–205.
29. Henderson, K. M., McNatty, K. P., O'Keeffe, L. E., Lun, S., Heath, D. A. & Prisk, M. D. (1987) *J. Reprod. Fertil.* **81,** 395–402.
30. McNatty, K. P., Lun, S., Hudson, N. L. & Forbes, S. (1990) *J. Reprod. Fertil.* **89,** 553–563.
31. Yi, S. E., Daluiski, A., Pederson, R., Rosen, V. & Lyons, K. M. (2000) *Development (Cambridge, U.K.)* **127,** 621–630.
32. Wang, T., Donahoe, P. K. & Zervos, A. S. (1994) *Science* **265,** 674–676.
33. Wang, T., Li, B. Y., Danielson, P. D., Shah, P. C., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T. & Donahoe, P. K. (1996) *Cell* **86,** 435–444.
34. Chen, Y. G., Liu, F. & Massague, J. (1997) *EMBO J.* **16,** 3866–3876.
35. Galloway, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., McLaren, R. J., Luiro, K., Dodds, K. G., Montgomery, G. W., Beattie, A. E., Davis, G. H. & Ritvos, O. (2000) *Nat. Genet.* **25,** 279–283.
36. Aaltonen, J., Laitinen, M. P., Vuojolainen, K., Jaatinen, R., Horelli-Kuitunen, N., Seppa, L., Louhio, H., Tuuri, T., Sjoberg, J., Butzow, R., Hovata, O., Dale, L. & Ritvos, O. (1999) *J. Clin. Endocrinol. Metab.* **84,** 2744–2750.