

The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality

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Abstract

The hyperprolificacy phenotype of Booroola ewes is due to the presence of the FecB^B allele at the FecB locus, recently identified as a single amino acid substitution (Q249R) in the bone morphogenetic protein (BMP) type-IB receptor (BMPR1B), and is associated with a more precocious differentiation of ovarian granulosa cells (GCs). To evaluate the consequences of the Booroola mutation on BMPR1B functions, the action of ligands of the transforming growth factor- β (TGF β)/BMP family that act through (growth and differentiation factor-5, BMP-4) or independently of (activin A, TGF β -1) BMPR1B were studied on primary cultures of GCs from homozygous FecB⁺ and FecB^B ewes. All the tested TGF β /BMP family ligands inhibited progesterone secretion by FecB⁺ GCs. Those inhibitory effects were lower for GCs from preovulatory (5–7 mm diameter) than from small antral follicles (1–3 mm diameter). The presence of the Booroola mutation

was associated with a 3- to 4-fold ($P < 0.001$) decreased responsiveness of GCs from FecB^B compared with FecB⁺ small follicles to the action of BMPR1B ligands. In contrast, TGF β -1 and activin A had similar inhibitory effects on progesterone secretion by GCs from FecB⁺ and FecB^B small follicles. No difference between genotypes was observed with GCs from preovulatory follicles. In transfection experiments with HEK-293 cells, co-expression of FecB⁺ BMPR1B and BMPR2 resulted in a 2.6-fold ($P < 0.01$) induction of the activity of a BMP-specific luciferase reporter construct by BMP-4. Interestingly, no response to BMP-4 was observed when cells were transfected with the FecB^B form of the BMPR1B receptor. Overall, these data strongly suggest that the Q249R mutation is associated with a specific alteration of BMPR1B signaling in hyperprolific Booroola ewes.

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Introduction

Booroola is an autosomal mutation identified on the basis of segregational studies on litter size and ovulation rate (Davis *et al.* 1982, Piper *et al.* 1985). The Booroola phenotype has a Mendelian pattern of segregation indicating that it is caused by a major gene, named FecB, which has additive effects on ovulation rate. The alleles are called FecB^B for the high prolificacy allele and FecB⁺ for the wild-type allele. In sexually mature ewes, the most consistent feature reported for FecB^B-carrier ewes is that ovarian follicles ovulate at a significantly smaller diameter than do those in non-carrier animals. In particular, based on the segregation of the ovulation rate in Merino flocks, the genotypes of the ewes are classified as homozygous non-carrier (FecB⁺/FecB⁺) with an ovulation rate of one or two and a size of ovulatory follicles of 7 mm diameter, heterozygous carriers (FecB^B/FecB⁺) with an ovulation rate of three or four and a size of ovulatory follicles of

4–5 mm diameter, and homozygous carriers (FecB^B/FecB^B) with more than five ovulations per cycle and a size of ovulatory follicles of 3–4 mm diameter (Davis *et al.* 1982). In the ovary, during follicular growth, the decrease in proliferative activity and the increase in the expression of the main markers of final follicular maturation, in particular luteinizing hormone receptor and aromatase, appear more precociously in granulosa cells (GCs) from homozygous FecB^B than in GCs from FecB⁺ ewes. Consequently, there are fewer GCs in FecB^B than in FecB⁺ preovulatory follicles, but the total number of GCs in preovulatory follicles per animal, as well as the ovarian secretion rate of steroids and inhibin, are similar in both genotypes (for a review see Baird & Campbell 1998).

Recently, the Q249R mutation in the coding sequence of the bone morphogenetic protein (BMP) type-IB receptor (BMPR1B) gene (*BMPR1B*) was found to be fully associated with the Booroola phenotype (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). BMPR1B

belongs to the family of receptors for transforming growth factor- β (TGF β)-like factors that is composed of several ligands such as TGF β , activins, BMPs, growth and differentiation factors (GDFs) and Mullerian inhibiting substance (Derynck & Feng 1997). Ligands of the TGF β /BMP family exert their biological effects through specific Ser/Thr-kinase transmembrane receptors, type I and type II. BMPR1B is one of the specific type I receptors for the subfamily of BMP and GDF ligands. Ligand binding induces the formation of heterotetramers of type I and type II receptors, leading to the activation of a specific intracellular signaling pathway by phosphorylation of Smad proteins (Derynck & Feng 1997, Miyazono 1999).

In sheep, we have previously shown that BMPR1B ligands, GDF-5 and BMP-4, inhibit progesterone secretion by ovine GCs isolated from small antral follicles (1–3 mm diameter) *in vitro* (Mulsant *et al.* 2001). Moreover, this inhibitory effect is attenuated with FecB^B GCs when compared with FecB⁺ GCs recovered from 1–3 mm diameter follicles in both genotypes, suggesting that Q249R substitution in BMPR1B may alter its function (Mulsant *et al.* 2001). However, as said above, it has been previously shown that terminal differentiation of GCs *in vivo* is acquired at a smaller follicular size in the FecB^B than in FecB⁺ genotype (Henderson *et al.* 1985, 1987, McNatty *et al.* 1986, Monniaux *et al.* 2000). So, it can be argued that the observed decrease in responsiveness of FecB^B GCs to BMPR1B ligands might be due to a more advanced cellular differentiation stage compared with FecB⁺ GCs of the same follicle size class, rather than to a direct effect of the mutation.

To address this issue, we have performed an overall study of the effects of different elements of the BMP/TGF β system (GDF-5, BMP-4, activin A and TGF β -1) on GCs from FecB⁺ and FecB^B follicles. In particular, we have studied the effect of GDF-5 and BMP-4 on steroidogenesis and proliferation of GCs isolated from ovine follicles of both genotypes at different stages of terminal follicular development. To assess the specificity of the differential effect observed with BMPR1B ligands on FecB⁺ and FecB^B GCs, we have studied the effects of TGF β -1 and activin A, which act independently of BMPR1B (Miyazono *et al.* 2001) and which inhibit progesterone production by GCs in different species (Shukovski & Findlay 1990, Miro *et al.* 1991, Kubota *et al.* 1994, Ford & Howard 1997). Moreover, we have tested the consequences of the Q249R mutation on BMPR1B signaling function in transient transfection experiments of BMP-responsive human epithelial kidney cells, HEK-293.

Materials and Methods

Animals

Cyclic FecB^B and FecB⁺ Mérimos d'Arles ewes (genotyped as described (Mulsant *et al.* 2001)) 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 castration 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).

Granulosa cell cultures

Briefly, in each independent culture experiment, ovarian follicles from four FecB^B and four FecB⁺ ewes were quickly dissected, pooled and classified according to size (i.e. small antral follicles, 1–3 mm in diameter; medium follicles, 3.5–4.5 mm; and large follicles, 5–7 mm) and genotype. Large follicles were found only in the FecB⁺ genotype. Large follicles from FecB⁺ ewes and medium follicles from FecB^B ewes were classified as preovulatory follicles. GCs were recovered from each class size follicles as previously described (Monniaux & Pisselet 1992). FecB^B and FecB⁺ GCs suspensions were seeded at 100 000 viable cells/well in 96-well plates and cultured for 96 h at 37 °C with 5% CO₂ in serum-free McCoy's 5a medium (Sigma, L'Isle d'Abeau Chesnes, France) containing insulin-like growth factor-I (IGF-I) (10 ng/ml; Ciba-Geigy, Saint-Aubin, Switzerland), according to a previously described method (Campbell *et al.* 1996). Cultures were performed with or without various concentrations (1, 5, 10 or 50 ng/ml) of dimeric recombinant human GDF-5 (Biopharm GmbH, Heidelberg, Germany), recombinant human BMP-4 (Genetics Institute, Inc., Cambridge, MA, USA), recombinant human activin A (National Hormone Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA) or 0.2, 1, 5 or 10 ng/ml recombinant human TGF β -1 (Sigma). Monomeric recombinant human GDF-5 (50 ng/ml; Biopharm) was used as a negative control. Each combination of treatments was tested in triplicate in each of four independent culture experiments. Culture media were partially replaced (180 of 250 μ l) at 48 h. Media conditioned between 48 and 96 h of culture were collected at 96 h and stored at –20 °C prior to progesterone assay. At the end of the culture, the number of cells per well was estimated after trypsinization by counting an aliquot of each resulting cell suspension with a hemacytometer under a phase contrast microscope.

Progesterone RIA

Progesterone amounts (ng/50 000 cells) in the culture media from each experiment were measured by RIA in

the same assay as described by Saumande (1991). The limit of detection of the assay was 12 pg/tube and the intra-assay coefficient of variation was 10%.

Detection of cytochrome P450 side chain cleavage (P450_{scc})

GCs from FecB⁺ ewes were cultured as described above. Each combination of treatments was tested in three independent cultures. After 96 h of culture, media were removed and GCs were resuspended in lysis buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol). Protein concentration was determined by a colorimetric assay (BC Assay Kit; Uptima Interchim, Montluçon, France). The protein samples (60 µg) were fractionated using SDS-PAGE in 12% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France). The same samples were run in duplicate, half of the membrane was used for P450_{scc} detection and the second half for actin detection, used as loading control. P450_{scc} was detected using a rabbit polyclonal antibody raised against P450_{scc} (dilution 1/1000; Chemicon Euromedex, Mundolsheim, France), followed by incubation with peroxidase conjugated anti-rabbit IgG (dilution 1/20 000; Interchim, Montluçon, France). Actin was revealed using a mouse monoclonal anti-actin antibody (dilution 1/1000; Sigma) followed by incubation with peroxidase-conjugated anti-mouse IgG (dilution 1/20 000, Bio-Rad, Marnes-la-Coquette, France). The protein bands were detected using ECL detection reagents (Amersham Pharmacia Biotech, Orsay, France).

Cell proliferation

In six independent cultures for both genotypes, proliferation was assessed by studying [³H]thymidine (0.25 µCi/ml (1 Ci=37 GBq); DuPont de Nemours, Les Ulis, France) incorporation for 2 h by GCs from small follicles, after 48 h of culture in the absence or in presence of GDF-5 (50 ng/ml), BMP-4 (50 ng/ml) or IGF-I (10 ng/ml) as a positive control. The labeling index (percentage of [³H]thymidine-labeled cells) was determined as described (Monniaux *et al.* 2000).

Plasmids and transient transfection

The FecB⁺ and FecB^B BMPR1B expression plasmids were generated using RT-PCR as described (Mulsant *et al.* 2001) and cloning of the PCR products into pSG5 vector (Stratagene, Amsterdam, The Netherlands). The presence of the Booroola mutation was verified by sequencing. pCMV-BMPR2, a human BMP receptor type II expression plasmid (Liu *et al.* 1995) and 12 GCCG-lux, a BMP-specific luciferase reporter construct (Kusanagi *et al.* 2000) were kindly provided by J Massagué and M Kawabata respectively.

Human epithelial kidney HEK-293 cells were seeded at 40 000 cells/well in 48-well plates and cultured for 24 h in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 3 mM glutamine and antibiotics (10 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml Fungizone). Then, plasmids and reporter gene (200 ng of each construct/well) were transiently transferred to cells using Dac30 transfection reagent for 3 h with a DNA/Dac30 ratio of 1/2 (w/w) as specified by the manufacturer (Eurogentec SA, Seraing, Belgium). After transfection, cells were starved in serum-free DMEM and incubated for 24 h with or without 100 ng/ml BMP-4 (Genetics Institute) before luciferase assays (Promega, Charbonnières, France). Each combination of plasmids was tested in triplicate in each culture and in three independent cultures.

Data analysis

All experimental data are presented as means ± S.E.M. For results of progesterone secretion, important variations were observed between independent cultures. Accordingly, data were expressed as percentages of the amount of progesterone secreted by 50 000 cells cultured in the absence of TGFβ/BMP family ligands. Absolute amounts of progesterone secreted in basal condition were as follow: FecB⁺ small follicles = 73.1 ± 18.3 ng (*n*=8); FecB⁺ medium follicles = 10.3 ± 5.5 ng (*n*=4); FecB⁺ preovulatory follicles = 86.6 ± 39.2 ng (*n*=8); FecB^B small follicles = 66.5 ± 12.5 ng (*n*=8); FecB^B preovulatory follicles = 137.2 ± 62.5 ng (*n*=8). For each follicle size class, or each genotype, the effects of ligands on progesterone secretion were analyzed using two-way ANOVA in order to appreciate the 'ligand effect' as well as the 'culture effect'. For comparison of the effects of TGFβ/BMP ligands between follicle sizes or between genotypes, means were compared using three-way ANOVA to allow for 'ligand effect', 'culture effect' and 'follicle size effect' or 'genotype effect'. For luciferase assays, in basal as well as in BMP-induced conditions, the effect of different combinations of plasmids was assessed by two-way ANOVA to allow for 'culture effect' and 'plasmid effect'. The effect of BMP-4 for each combination of plasmids was assessed by two-way ANOVA to allow for 'culture effect' and 'BMP-4 effect'. Post-hoc comparisons were performed with the Scheffé and Newman-Keuls tests. For the thymidine labeling index, comparisons of means between control and ligand-treated conditions were performed using paired *t*-tests for each genotype. For all analysis, differences with *P*>0.05 were considered as not significant.

Results

Responsiveness of GCs from FecB⁺ ewes to BMPR1B ligands: effect of follicular size

Addition of increasing doses of GDF-5 or BMP-4 led to a dose-dependent inhibition of progesterone secretion by

GCs from small, as well as medium and preovulatory FecB^+ follicles (Fig. 1A). In contrast, the monomeric form of GDF-5, used as a negative control, had no effect at the highest dose tested (50 ng/ml). Moreover, accumulation of immunoreactive P450_{scc} decreased in the presence of BMP-4 (Fig. 1B). So, inhibition of progesterone secretion by BMP-4 might be, at least partly, due to inhibition of P450_{scc} accumulation, a rate-limiting step in progesterone synthesis.

GCs from medium and preovulatory follicles were less responsive to GDF-5 and BMP-4 action than those from small follicles. Indeed, the first dose of GDF-5 that was effective in inhibiting progesterone secretion was 1 ng/ml with GCs from small follicles compared with 10 ng/ml with GCs from larger follicles (Fig. 1A). Moreover, at the highest dose used (50 ng/ml), GDF-5 and BMP-4 actions were 2- and 3-fold more effective with GCs from small follicles compared with more mature GCs respectively ($P < 0.05$). Overall, these results indicate that the effects of *BMPR1B* ligands on GCs decreased during terminal follicular development in ewes.

Comparison of FecB^+ and FecB^B genotypes for responsiveness to *BMPR1B* ligands

GDF-5 and BMP-4 (50 ng/ml) exhibited inhibitory actions on progesterone secretion by FecB^B GCs from both small follicles (as shown previously (Mulsant *et al.* 2001)) and preovulatory follicles (Fig. 2A). Monomeric GDF-5 (50 ng/ml) was without effect on progesterone production. As previously shown as well, GCs from small follicles were 3- and 4-fold less responsive to *BMPR1B* ligands in FecB^B than in FecB^+ genotype (Fig. 2A). In sharp contrast, no difference in responsiveness to *BMPR1B* ligands was observed on GCs from FecB^+ and FecB^B preovulatory follicles.

BMP-4 induced a significant increase ($P < 0.05$) in the proliferation rate of GCs from FecB^+ small follicles as assessed by thymidine labeling index (Fig. 2B), but had no effect on GCs from FecB^B small follicles. GDF-5, probably due to its lower activity compared with BMP-4 as seen on

progesterone secretion (Fig. 1A), exerted no significant effect on GC proliferation in FecB^+ and FecB^B genotype. The increase of GC proliferation by IGF-I ($P < 0.05$), used as a positive control, was not different in FecB^+ and FecB^B genotypes (Fig. 2B).

In conclusion, responsiveness to *BMPR1B* ligands was altered in FecB^B compared with FecB^+ GCs from small antral but not preovulatory follicles.

Specific alteration of *BMPR1B* signaling in FecB^B GCs

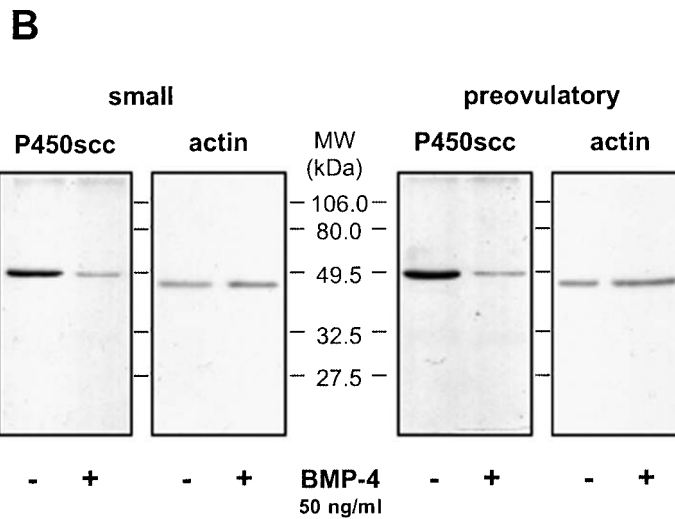
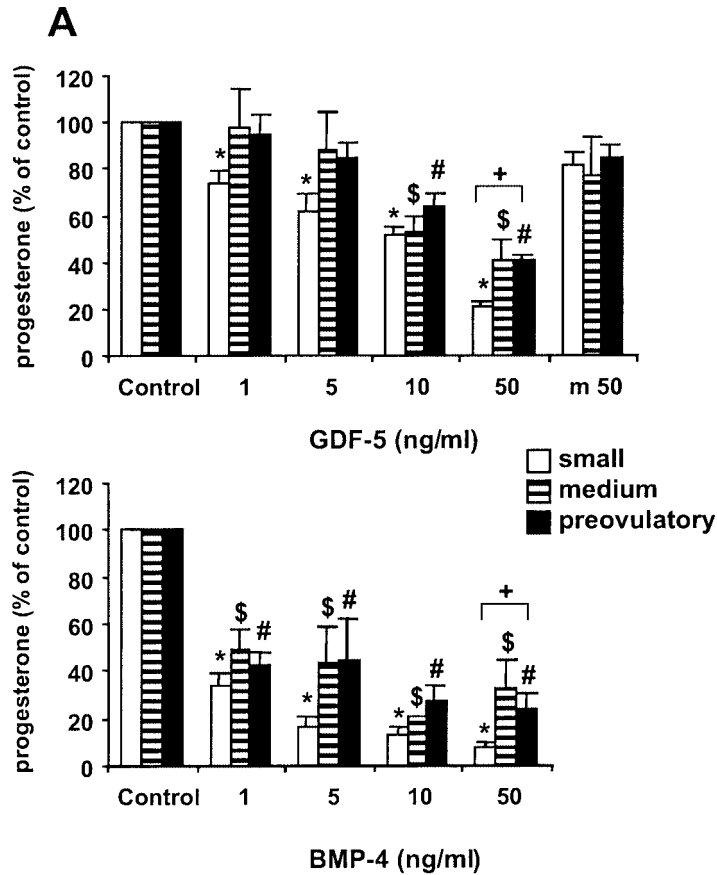
Several studies have shown that terminal differentiation of GCs is acquired at a smaller follicular size in FecB^B than in FecB^+ genotypes (Henderson *et al.* 1985, 1987, McNatty *et al.* 1986, Monniaux *et al.* 2000). So it can be argued that the decrease in responsiveness of GCs from FecB^B small follicles might be caused by their more advanced differentiation stage compared with FecB^+ GCs when isolated from the same follicle size class. To address this issue, we studied the action of two other factors of the TGF β family, activin A and TGF β -1 on GCs of both genotypes. Indeed, biological effects of activin A and TGF β -1 are independent of the *BMPR1B* receptor (Miyazono *et al.* 2001) and both molecules are also known to inhibit progesterone secretion (Shukovski & Findlay 1990, Miro *et al.* 1991, Kubota *et al.* 1994, Ford & Howard 1997). As expected, activin A and TGF β -1 exerted strong inhibitory effects on progesterone secretion by ovine GCs *in vitro* (Fig. 3). These effects were dose-dependent and occurred similarly in both genotypes for GCs from small as well as preovulatory follicles. Moreover, in contrast to BMP-4, activin A and TGF β -1 had no effect on the proliferation rate of GCs from small follicles of both genotypes (data not shown). Overall, these results indicate that the signaling pathway of BMP factors, but not TGF β -1 or activin A, was specifically altered in FecB^B GCs from small follicles.

As for *BMPR1B* ligands, preovulatory follicles appeared less sensitive to activin A and TGF β -1 action than small follicles. Indeed, the first dose of activin A which was effective in inhibiting progesterone secretion was 10 ng/ml with GCs from small follicles ($P < 0.05$) compared with

Figure 1 (opposite) Effects of GDF-5 and BMP-4 on steroidogenesis by FecB^+ GCs from different follicles size classes *in vitro*. GCs from small antral (1–3 mm in diameter), medium (3.5–4.5 mm) and preovulatory (5–7 mm) FecB^+ follicles were cultured for 96 h in serum-free conditions. (A) Effects on progesterone secretion. Cultures were performed in the absence (control) or presence of different concentrations of dimeric GDF-5 or BMP-4, or 50 ng/ml monomeric GDF-5 as negative control (m 50). Each combination of treatments was tested in triplicate in each of four independent experiments. Results represent progesterone secretion by 50 000 GCs between 48 and 96 h of culture. Data are expressed as percentages (means \pm S.E.M.) of the amount of progesterone secreted by cells cultured in control conditions. * $P < 0.05$, small follicles, ligand treated vs control; [§] $P < 0.05$, medium follicles, ligand treated vs control; [#] $P < 0.05$, preovulatory follicles, ligand treated vs control; ^{*} $P < 0.05$, small vs medium or preovulatory follicles. (B) Representative Western immunoblotting analysis of GCs whole cell extract using anti-P450_{scc} antibody. GCs from FecB^+ small or preovulatory follicles were cultured with or without BMP-4 (50 ng/ml) for 96 h. At the end of the culture, GCs were resuspended in lysis buffer and submitted to immunoblotting as described in Materials and Methods. Actin was used as gel loading control. Positions of molecular weight standards are indicated (MW).

50 ng/ml with GCs from preovulatory follicles ($P < 0.05$). Moreover, at the same dose used, TGF β -1 was 2- to 3-fold more active with GCs from small follicles compared with preovulatory follicles (Fig. 3).

Thus, as for BMPR1B ligands, activin A and TGF β -1 responsiveness decreased during terminal follicular development in ewes. However, in contrast, it was not affected by the presence of the Booroola mutation.



Consequence of the Q249R substitution on *BMPR1B* signaling

By using the HEK-293 cell line, we investigated the capacity of the Booroola form of the *BMPR1B*(QR) receptor to trigger the BMP-4 signaling pathway. The HEK-293 cell line was transiently transfected with a BMP-specific reporter construct (12GCCG-lux) (Kusanagi *et al.* 2000), with or without vectors encoding the human *BMPR2*, and with either the wild-type form of ovine *BMPR1B* or the *BMPR1B*(QR). After transfection, HEK-293 cells were treated for 24 h with or without 100 ng/ml BMP-4. The results (Fig. 4) show that HEK-293 cells were endogenously BMP-responsive and that this response was not affected by overexpression of BMP receptor type II alone. When *BMPR2* and *BMPR1B* were co-expressed to allow for newly formed receptor complexes, BMP-4 enhanced luciferase activity by 2.6-fold over basal levels. In the presence of *BMPR1B*(QR) receptor, basal luciferase activity was significantly increased ($P < 0.01$) and no activation of luciferase activity was observed in the presence of BMP-4. Thus, signaling activity of overexpressed *BMPR1B* was altered by the Booroola mutation.

Discussion

In this study and for the first time, we have performed an overall analysis of the effects of different elements of the TGF β /BMP system on ovine GCs from different follicular size classes *in vitro*. Moreover, we have investigated the functional consequences of the Q249R mutation in the *BMPR1B* receptor of Booroola ewes. Our data led to the conclusion that: (i) BMP/TGF β factors exerted a strong inhibitory effect on progesterone secretion by GCs from small antral follicles in the ewe – this inhibitory effect decreasing during terminal follicular growth; (ii) the responsiveness to *BMPR1B* ligands was altered in FecB^B compared with FecB⁺ GCs from small antral but not preovulatory follicles; (iii) the TGF β -1 and activin A inhibition of progesterone secretion was similar in GCs from FecB⁺ and FecB^B genotypes, strongly suggesting that *BMPR1B* signaling was specifically altered in Booroola follicles; and (iv) the Booroola mutation Q249R was able to directly alter the signaling pathway triggered by *BMPR1B* receptor. Overall, these data strongly suggest that the hyperproliferative phenotype of Booroola ewes is associated with an altered *BMPR1B*-dependent signaling.

In the rat, Shimasaki *et al.* (1999) have shown that BMP-4 and BMP-7 inhibit progesterone but enhance estradiol secretion by GCs. In our experimental conditions, secretion of estradiol by ovine GCs *in vitro* is low and not stimulated by BMPs. So, our ovine model does not allow assessment of the effects of the Q249R mutation on estradiol secretion. Nevertheless, *in vivo*, it is proposed that BMP factors would impair premature luteinization of

ovarian follicles (Shimasaki *et al.* 1999). The present data have shown a decrease in the responsiveness of GCs from preovulatory, compared with small, follicles to the effects of *BMPR1B* ligands, as well as TGF β -1 and activin A, suggesting that the anti-luteinizing effects of these factors decrease during terminal follicular growth. Moreover, from our results, it is strongly suggested that Q249R substitution is associated with a loss of responsiveness of *BMPR1B* receptor to BMP ligands. Then, in Booroola ewes, Q249R substitution would impair the anti-luteinizing function of *BMPR1B* pathway in growing follicles, leading to an advanced differentiation of GCs and an advanced maturation of follicles.

Surprisingly, the differential effect of BMP-4 and GDF-5 between genotypes was observed only on GCs isolated from small but not preovulatory follicles. This result could be partly explained by some redundancy of BMP receptors. In particular, *BMPR1A*, another receptor for BMP-4 and GDF-5 (ten Dijke *et al.* 1994, Nishitoh *et al.* 1996, Yi *et al.* 2000), was also shown to be expressed in ovine GCs (Souza *et al.* 2002). One could hypothesize that expression and/or activation of *BMPR1B* and *BMPR1A* would be predominant in small and preovulatory follicles respectively. Then, the Booroola mutation would have an effect only on small follicles. However, by *in situ* hybridization or immunohistochemistry, Wilson *et al.* (2001) and Souza *et al.* (2002) did not report any striking difference in expression of *BMPR1B* in the different categories of ovine follicles. Moreover, by RT-PCR, we did not observe any difference in expression of these two receptors between GCs isolated from ovine small or preovulatory follicles (S Fabre, unpublished observations). Alternatively, one could also hypothesize that the Q249R mutation would have a consequence on the functionality of *BMPR1B* only in the presence of another partner that would be expressed and/or functional in small but not preovulatory follicles. The existence of such a factor and its consequences on the signaling pathway should now be investigated.

Whether the Booroola mutation leads to a partial or a complete alteration of *BMPR1B* function remains unknown. In GCs from FecB^B small follicles, inhibition of progesterone secretion by *BMPR1B* ligands was largely reduced but still existed, suggesting a possible partial loss-of-function mutation. However, the residual response of GCs to GDF-5 and BMP-4 could be due to *BMPR1A* signaling. From transient transfection experiments of HEK-293 cells, the Q249R mutation seems to entirely abrogate the ability of *BMPR1B* to respond to the BMP-4 stimulation, suggesting a complete loss of responsiveness of FecB^B *BMPR1B* to its ligands. Nevertheless, in these experiments, we have also observed a significant increase in the luciferase basal activity triggered by the FecB^B form of *BMPR1B*, indicating that the Q249R substitution could act as a constitutively active mutation in *BMPR1B*. If this last observation is physiologically relevant, then

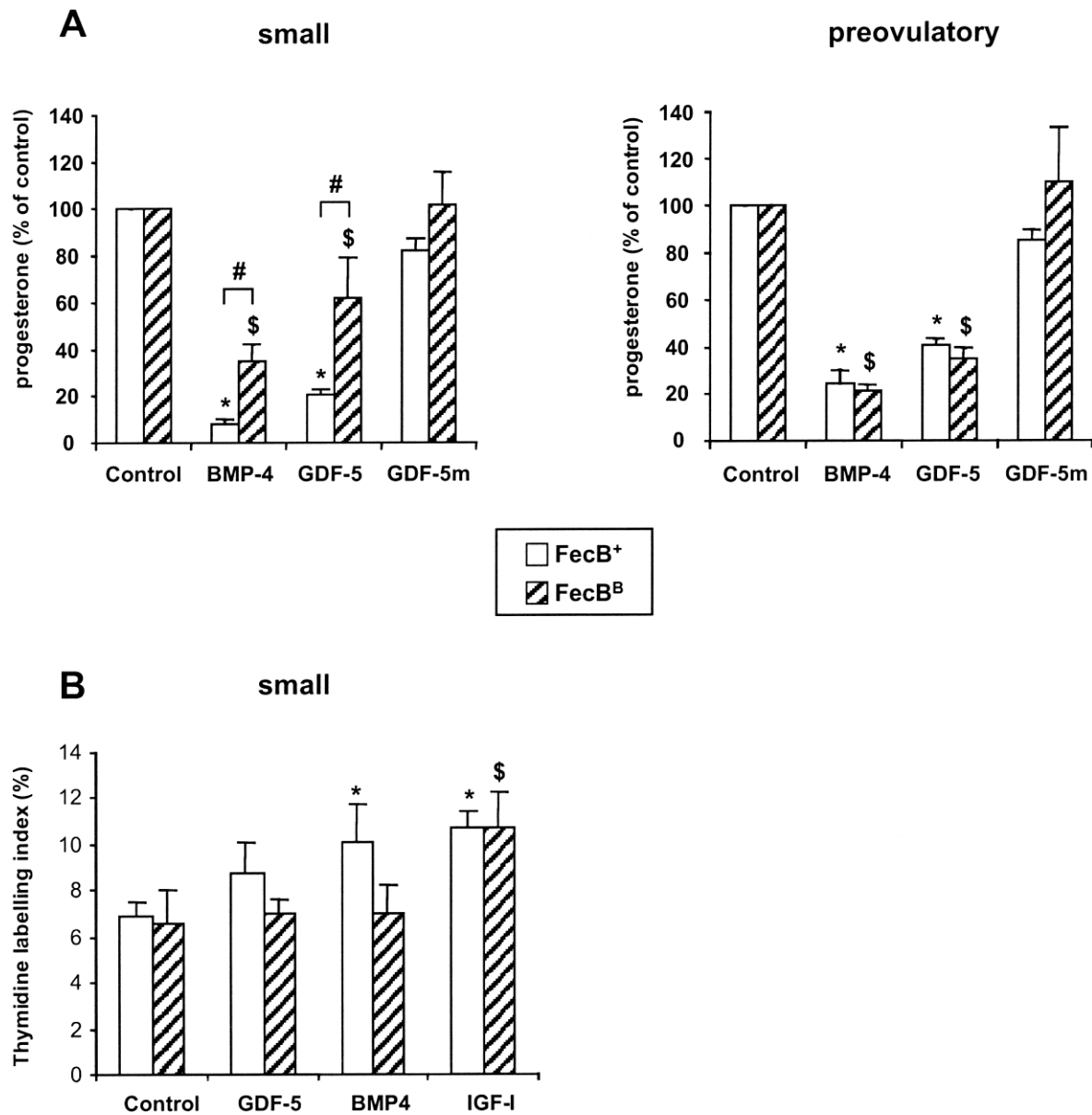


Figure 2 Effect of the Booroola mutation on GCs' responsiveness to BMPR1B ligands *in vitro*. GCs were collected from FecB⁺ (open bars) and FecB^B (hatched bars) small antral (1–3 mm in diameter) and preovulatory (FecB⁺: 5–7 mm, FecB^B: 3.5–4.5 mm) follicles. (A) Progesterone secretion. GCs were cultured for 96 h in serum-free conditions in the presence or absence (control) of 50 ng/ml human recombinant dimeric GDF-5 or BMP-4, or monomeric GDF-5 as negative control (GDF-5m) in the culture medium. Each combination of treatments was tested in triplicate in each of four independent experiments. Results represent progesterone secretion by GCs between 48 and 96 h of culture. Data are expressed as percentages (means ± S.E.M.) of the amount of progesterone secreted by cells cultured in the absence of BMPR1B ligands. (B) Cell proliferation. Data represent the thymidine labelling index (see Materials and Methods) of GCs from FecB⁺ and FecB^B small follicles after 48 h of culture in the absence or presence of GDF-5 (50 ng/ml), BMP-4 (50 ng/ml) or IGF-I (10 ng/ml). **P*<0.05, FecB⁺ ligand treated vs control; [§]*P*<0.05, FecB^B ligand treated vs control; #*P*<0.05, FecB^B vs FecB⁺.

ovarian progesterone secretion of FecB^B ewes should be constitutively inhibited. BMPR1B is known to be expressed in ovine corpora lutea (Wilson *et al.* 2001, Souza *et al.* 2002) but physiological studies have never demon-

strated a significant difference in progesterone secretion of Booroola compared with wild-type ewes (Baird & Campbell 1998). In contrast to hyperprolificacy of Booroola ewes, *bmpr1b* - / - female mice are sterile,

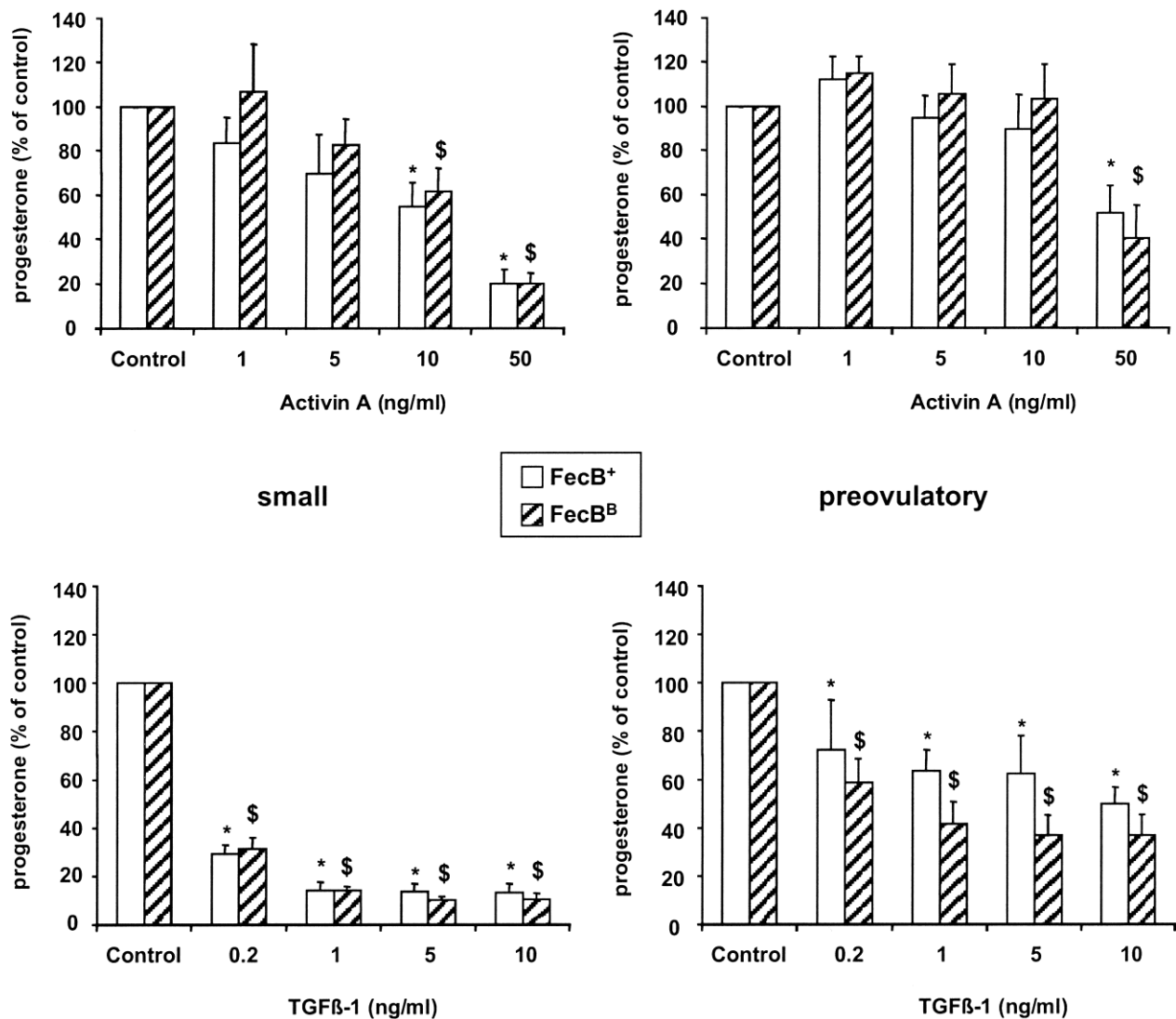


Figure 3 Effect of the *Booroola* mutation on GCs' responsiveness to activin A and TGF- β 1 *in vitro*. Experimental conditions were the same as in Fig. 2A. GCs from FecB⁺ and FecB^B small and preovulatory follicles were cultured in the absence (control) or presence of increasing amounts of recombinant human activin A (upper panels) or TGF- β 1 (lower panels). Data are expressed as percentages of the amount of progesterone secreted by cells cultured in the absence of TGF- β family ligands. * $P < 0.05$, FecB⁺ ligand treated vs control; \$ $P < 0.05$, FecB^B ligand treated vs control.

cumulus expansion and embryo implantation being dramatically altered (Yi *et al.* 2001). Actually, data on the effects of a natural point mutation in sheep and of an experimental deletion in the mouse are difficult to reconcile. In particular, consequences of inactivation of one or both copies of *bmp15* are completely different in mouse and sheep (Galloway *et al.* 2000, Yan *et al.* 2001). These discrepancies are probably due to species differences, and/or to the nature of the alteration (point mutation vs deletion). Nevertheless, further studies are needed to assess the degree of alteration of BMPR1B receptor in *Booroola* ewes.

The physiological ligands of BMPR1B remain to be determined in sheep ovary. BMP-4 and BMP-7 were shown to be expressed by follicular theca cells in the rat (Shimasaki *et al.* 1999). BMP-3 has been shown to be expressed by human granulosa-luteal cells (Jatinen *et al.* 1996), and the oocyte expresses BMP-6, GDF-9 and BMP-15 (for a review see Elvin *et al.* 2000). Preliminary RT-PCR experiments indicate the presence of mRNA for BMP-2, BMP-4, BMP-6, BMP-7, GDF-9 and BMP-15 in antral ovine whole follicles (data not shown). Otsuka *et al.* (2001) have suggested recently that among the BMP ligands expressed by the follicle, BMP-6 repre-

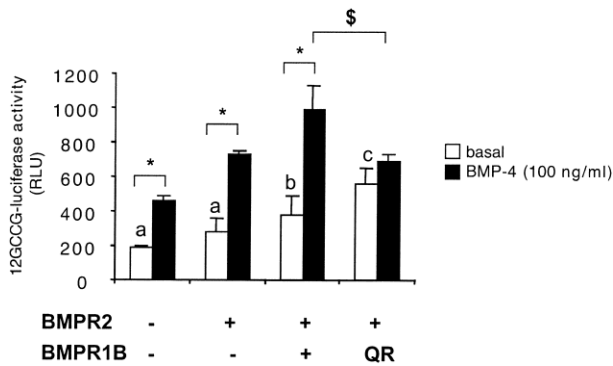


Figure 4 Effect of the Booroola mutation on BMPR1B signaling activity. HEK-293 cells growing in 10% FCS were transiently transfected with the 12 GCCG-lux reporter construct and different combinations of plasmids expressing human BMPR2, ovine BMPR1B with or without the Q249R mutation (QR). After transfection, cells were cultured 24 h in serum-free conditions in the absence (basal) or presence of 100 ng/ml BMP-4 before luciferase assays. Luciferase activity is expressed as relative light units (RLU). Data represent means ± S.E.M. of measurements performed in three independent experiments. * $P < 0.05$, BMP-4 vs basal; † $P < 0.01$. a, b, c denote significant statistical differences in basal activities, $P < 0.05$.

sents the more likely candidate ligand for BMPR1B signaling. To test the hypothesis that one of these oocyte-derived BMP factors acts through BMPR1B, it would be of great interest to compare their biological effects on GCs from FecB⁺ and FecB^B small antral follicles.

All the factors tested, acting through (BMP-4 and GDF-5) or independently of (TGFβ-1 or activin A) BMPR1B receptor, exerted a strong inhibitory action on progesterone secretion by ovine GCs *in vitro*. BMP-7 and the three oocyte-derived BMP family members also strongly inhibit progesterone secretion by follicle-stimulating hormone-treated rodent granulosa cells *in vitro* (Shimasaki *et al.* 1999, Otsuka *et al.* 2000, 2001, Vitt *et al.* 2000). This suggests a certain redundancy of the anti-luteinizing action of the TGFβ/BMP system in the ovary *in vivo* and raises the question of the relative importance of each pathway for controlling terminal follicular maturation and ovulation rate. Recent findings have shown that hyperprolificacy of Inverdale and Hanna ewes was due to loss-of-function mutations in the *BMP15* gene (Galloway *et al.* 2000). Overall, the Booroola, Inverdale and Hanna genetic models of hyperprolificacy in sheep strongly suggest that the BMP system plays a key role in folliculogenesis and that a decrease in BMP signaling positively regulates the number of ovulatory follicles.

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