Effect of follicle size and of the *Fec^B* Booroola gene on oocyte function in sheep

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Booroola ewes have a major gene that affects ovulation rate. Gene expression has consequences on ovarian somatic cells but it is unknown whether it also affects germ cells in the adult ovary. Hence, the present study examined (1) whether oocyte growth was similar in *Fec^BFec^B* and *Fec⁺Fec⁺* oocytes during preantral and antral follicular growth, (2) whether the patterns of proteins neosynthesized by oocytes of these two genotypes were identical, (3) whether the ability of the oocytes to resume meiosis was unaffected by genotype and (4) whether, after IVF, oocytes from both genotypes could develop to the blastocyst stage at similar rates. Histological examination of the respective sizes of the oocyte and of the follicle demonstrated that oocytes were larger in *Fec^BFec^B* versus *Fec⁺Fec⁺* preantral follicles. Resolution of the proteins neosynthesized by *Fec^BFec^B* and *Fec⁺Fec⁺* oocytes by one-dimensional PAGE and image analysis demonstrated that quantitative (but not qualitative) differences could be observed between genotypes for bands at 74, 59, 35 and 25 kDa. In addition, a genotype by oocyte size interaction was detected for two additional bands at 45 and 43 kDa. After 24 h of culture *in vitro* in TCM-199 plus 100 ng ml⁻¹ FSH plus 10% sheep follicular fluid, oocytes from *Fec^BFec⁺* follicles gained the ability to resume meiosis at a smaller size and a higher proportion of them reached metaphase II irrespective of the size class studied compared with *Fec⁺Fec⁺* follicles. In addition, the developmental rate of eggs after IVF was also affected by follicle size and genotype, since *Fec^BFec⁺* oocytes originating from 1.0–3.5 mm follicles had a greater ability ($P < 0.05$) to develop to the blastocyst stage than *Fec⁺Fec⁺* oocytes. It is concluded that the *Fec^B* gene, in addition to its effects on granulosa cell maturation, also affects oocyte development and function. Whether these alterations are related requires further investigation.

Introduction

Booroola ewes have a major gene *Fec^B* that influences their ovulation rate (for review see Montgomery *et al.*, 1992). Homozygotes (*Fec^BFec^B*), heterozygotes (*Fec^BFec⁺*) and non carriers (*Fec⁺Fec⁺*) are segregated on the basis of their ovulation rates (≥ 5 , 3–4 and 1–2, respectively) (Davis *et al.*, 1982) or more sophisticated statistical methods (Elsen and Le Roy, 1991).

The physiological effects of the *Fec^B* gene have been extensively characterized. Ovulatory follicles of *Fec^BFec^B* ewes are smaller and contain fewer granulosa cells (Driancourt *et al.*, 1985; McNatty *et al.*, 1986). Follicles from *Fec^BFec^B* ewes mature at a smaller size, as shown by their ability to produce oestradiol (McNatty *et al.*, 1986), to produce cAMP in response to a gonadotrophin challenge (Henderson *et al.*, 1987) and in their pattern of insulin-like growth factor binding proteins (IGFBPs) (P. Monget and D. Monniaux, unpublished). However, the

mechanisms responsible for this smaller size and earlier maturation have not been clarified. Although a role for the high FSH concentrations observed in *Fec^BFec^B* ewes (McNatty *et al.*, 1987) cannot be discounted, there is evidence that the high ovulation rate of the *Fec^BFec^B* ewes may be reached by an alteration in intraovarian regulation (Fry *et al.*, 1988; McNatty *et al.*, 1993). This could be achieved via a change in the autocrine–paracrine regulations between granulosa and theca cells or, alternatively, via a change in the dialogue between the oocyte and the surrounding somatic cells. Indeed, the oocyte has been shown to be able to affect granulosa cell proliferation and differentiation (Vanderhyden *et al.*, 1991, 1993; Eppig *et al.*, 1997).

Hence, the aims of this study were to compare the features of the oocytes of *Fec^BFec^B* or *Fec^BFec⁺* ewes those of with *Fec⁺Fec⁺* ewes. Specific endpoints measured were (1) oocyte growth in preantral follicles, (2) protein synthesis by the oocyte in small–large antral follicles, (3) the ability to resume meiosis in small–large antral follicles, and (4) the ability of these oocytes to develop to the blastocyst stage after *in vitro* fertilization (IVF).

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Materials and Methods

Animals

Five $Fec^B Fec^B$, 67 $Fec^B Fec^+$ and 75 $Fec^+ Fec^+$ Merino d'Arles ewes were assigned a specific genotype after statistical analysis (Elsen and Le Roy, 1991) of several records of ovulation rate. All ewes had their oestrous cycles synchronized by the insertion of progestagen impregnated sponges (Chronogest, Intervet, France). On day 12 (day 0: day of oestrus), all ewes were slaughtered and their ovaries were collected.

Experiment 1: pattern of oocyte growth in preantral follicles

Fragments of ovaries of five $Fec^B Fec^B$ and five $Fec^+ Fec^+$ ewes devoid of macroscopically visible follicles were fixed in Bouin Hollande's solution. They were then dehydrated, embedded in wax and sectioned at a 10 μ m thickness. All sections were mounted and stained with Shorr's stain (Centola, 1982). On the section where the oocyte nucleus was present, oocyte diameter (two orthogonal diameters) and follicle diameter (two orthogonal diameters) were measured.

Experiment 2: protein patterns of small–large antral follicles

The remainder of the ovaries of the five $Fec^B Fec^B$ and five $Fec^+ Fec^+$ ewes used in Expt 1 was used in this experiment. All follicles that could be detected macroscopically and microscopically were dissected carefully with watchmaker forceps. After careful measurement of their diameter, all follicles were split in two to collect (1) the oocyte–cumulus complex and (2) the follicular wall.

Each cumulus–oocyte complex was placed in a sterile Eppendorf tube containing 100 μ l of PBS plus BSA (0.5 g l⁻¹) in which 13 μ l of [³⁵S]methionine (NEN, Les Ulis) was added to reach a final concentration of 74 MBq ml⁻¹ as used by Crosby *et al.* (1981) and Kastrop *et al.* (1990). Oocytes were incubated at 37°C for 3 h. This duration was selected to ensure that only oocytes at the germinal vesicle (GV) stage were studied. In sheep, oocytes remain at the GV stage for the first 6 h of culture and undergo germinal vesicle breakdown (GVBD) between 7 h and 9 h of culture (Moor and Crosby, 1985). At the end of incubation, the cumulus–oocyte complexes were rinsed twice in PBS without BSA and then transferred to a Petri dish where cumulus cells were removed by repeated pipetting. Oocyte diameter was measured (excluding the zona pellucida) before the oocytes were transferred to lysis buffer (0.125 mol Tris–HCl l⁻¹, 0.35 mol SDS l⁻¹, glycerol 15%). All samples were heated for 1 min at 90°C and then stored at –20°C. Radioactivity incorporated in each sample was measured and 50 000 d.p.m. of each sample was loaded on 10% acrylamide gels. Electrophoresis was conducted according to Roberts *et al.* (1984). Gels were stained with Coomassie blue R 250, destained in acetic acid, ethanol and water, soaked in water, equilibrated for 30 min in sodium salicylate and dried. Fluorographs were prepared with Kodak XAR X-ray films and exposed for 3 weeks at –70°C. Each fluorograph was submitted to qualitative and quantitative analysis using Kepler Software (Large Scale Biology, Rockville, TN) (Driancourt *et al.*,

1996). Gels were digitized via a charge coupled device camera (Kodak, Eikonix 1412) serving as scanner. All bands present in each sample were identified and a master lane summarizing the bands present at least once in a sample was generated. The darkness of each band was then measured. The frequencies of bands in specific groups were compared by two way ANOVA (genotype effect, oocyte size effect and their interaction). Since the aim was to characterize individual oocytes and since many oocytes had not incorporated 200 000 d.p.m. (the load required for two-dimensional electrophoresis), this latter approach was not attempted.

Follicular walls were used to assess the interactions between follicle atresia and the pattern of oocyte proteins. Because oocyte function is likely to be affected only during advanced stages of atresia (Driancourt *et al.*, 1991), a marker of advanced atresia had to be found. Amounts of 17 α hydroxylase appeared to be suitable since, in sheep (Huet *et al.*, 1997), decreased amounts are associated with advanced atresia, while other steroidogenic enzymes are affected earlier during atresia (aromatase) or are not markedly affected (P450scc). The follicular walls were immediately immersed in hypotonic buffer (10 mmol KCl l⁻¹, 10 mmol Tris l⁻¹, 0.5 mmol EDTA l⁻¹, pH 7.2) in which protease inhibitors (1 μ mol phenyl methyl sulfonyl fluoride l⁻¹, 10 μ mol *N*- α -tosyl-L-lysine chloromethyl ketone (TLCK) l⁻¹ and 100 μ mol *N*-tosyl-L-phenylalanine (TPCK) l⁻¹) were added. After sonication for 30 s, the amounts of protein present in each sample were determined by the method of Bradford (1976). Equal amounts of protein from each follicle were then loaded on 10% acrylamide gels (Roberts *et al.*, 1984), and transferred by overnight electroblotting at 4°C on nitrocellulose membranes. Membranes were immunoblotted with polyclonal antibodies against 17 α -hydroxylase (kindly provided by I. J. Mason, University of Edinburgh). Immunoblotting and immunohistochemistry have shown this antibody to be specific (Lund *et al.*, 1988). Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) kit provided by Amersham (Les Ulis). The darkness of the band was quantified using the Kepler Software. Because the distribution of the darkness units was clearly bimodal, a threshold (10 000 darkness units) could be identified separating a group of follicles exhibiting high 17 α -hydroxylase (not in advanced atresia) from another exhibiting low 17 α -hydroxylase (in advanced and late atresia).

Experiment 3: nuclear maturation of oocytes of different sizes in $Fec^B Fec^+$ and $Fec^+ Fec^+$ genotypes

The ovaries of 26 $Fec^B Fec^+$ and 29 $Fec^+ Fec^+$ Merino d'Arles ewes slaughtered on day 12 of the cycle were dissected and follicles were separated into three groups (0.6–1 mm, 1.1–1.5 mm and >1.6 mm) to study oocyte nuclear maturation (nine replicates).

The oocytes were isolated by rupture of the follicular wall and only those surrounded by multilayer cumulus cells were matured *in vitro* (IVM) for 24 h in 0.5 ml TCM-199 (M199, Sigma, St Quentin Fallavier) supplemented with 10% sheep follicular fluid collected from medium size (4–5 mm) follicles during the breeding season and 100 ng oFSH ml⁻¹ (Cognie *et al.*, 1995). After IVM, the cumulus cells were removed

mechanically and oocytes were fixed and stained with Hoechst 33342, a DNA-fluorescent dye used to evaluate the meiotic stages reached in each group. Oocytes observed under the microscope were classified as GV, pro-metaphase I, metaphase I or metaphase II. A control group of 92 $Fec^B Fec^+$ and 38 $Fec^+ Fec^+$ oocytes was fixed and stained at the onset of IVM ($t = 0$) and the diameters of oocytes (excluding zona pellucida) were measured with an ocular micrometer attached to an inverted microscope to check whether oocyte size in large follicles was affected by the genotype.

Experiment 4: effects of FSH on the ability of the oocytes to develop to the blastocyst stage after maturation and fertilization in vitro

Ovaries of 43 $Fec^B Fec^+$ and 41 $Fec^+ Fec^+$ ewes were dissected and follicles were ranked into three groups (1–2 mm, 2.1–3.5 mm and > 3.5 mm) to study oocyte nuclear maturation (three replicates) and cytoplasmic maturation (seven replicates). *In vitro* matured oocytes were fertilized *in vitro* using frozen-thawed semen (Cognie *et al.*, 1991) to investigate the effect of the presence of FSH (100 ng oFSH ml⁻¹, batch 1766 III exhibiting a specific activity of 3.5 NIHFSHS1, a gift from Y. Combarnous, INRA, PRMD) in the maturation medium. Briefly, after separation of the motile spermatozoa using a Percoll buffered gradient and centrifugation at 500 g for 20 min, 1×10^6 spermatozoa ml⁻¹ were incubated with 10–30 oocytes in Brackett's defined medium buffered with Hepes and containing 20% heat-inactivated sheep serum at 38.5°C for 17 h.

The cleavage rate at day 2 after IVF and developmental competence using embryo culture for 7 days in synthetic oviductal fluid (SOF) medium (Takahashi and First, 1992) were determined in 338 $Fec^B Fec^+$ and 311 $Fec^+ Fec^+$ oocytes. Hatching rate (percentage of blastocysts that completely hatched) and the number of cells per embryo was assessed on day 7 of culture. Data were analysed by chi-square or Fisher's exact test, where appropriate.

Results

Experiment 1: pattern of oocyte growth in preantral follicles

Seventy three $Fec^B Fec^B$ and 61 $Fec^+ Fec^+$ follicles were present in the ovarian fragments studied and had oocyte and follicle size measured. When allocated to specific follicle size classes (< 50 µm, 51–100 µm, 101–150 µm, 151–200 µm and > 201 µm in diameter), the respective proportion of follicles of each genotype in these classes was ($Fec^B Fec^B$: 24.6, 46.6, 17.8, 4.1 and 6.8% versus $Fec^+ Fec^+$: 39.1, 28.8, 13.9, 5.0 and 13.2%, respectively).

Oocyte size in primordial follicles (that is, those with a diameter < 50 µm) was similar in both genotypes ($Fec^B Fec^B$: 28.1 ± 0.8 µm versus $Fec^+ Fec^+$: 28.6 ± 3.5 µm). In contrast, as follicles enlarged, oocytes were larger in $Fec^B Fec^B$ than in $Fec^+ Fec^+$ follicles (Fig. 1). For follicles 51–100 µm in diameter, oocyte diameter was 45.0 ± 1.5 µm versus 41.2 ± 1.7 µm in $Fec^B Fec^B$ and $Fec^+ Fec^+$, respectively ($P < 0.05$). For follicles 101–150 µm in diameter, oocyte diameter was 76.7 ± 3.9 and 59.6 ± 1.7 µm for $Fec^B Fec^B$ and $Fec^+ Fec^+$, respectively

Fig. 1. Comparative patterns of oocyte growth in preantral and small antral follicles belonging to follicles from $Fec^B Fec^B$ (◆) and $Fec^+ Fec^+$ (□) ewes.

($P < 0.05$). For follicles 151–200 µm in diameter, oocyte diameter was 106.7 ± 1.5 µm and 71.2 ± 3.0 µm for $Fec^B Fec^B$ and $Fec^+ Fec^+$, respectively ($P < 0.01$). The changes in the respective sizes of the oocyte (y) and of the follicle (x) during the preantral stage can be described by the following correlations ($Fec^B Fec^B$: $y = 0.58x + 3.78$, $Fec^+ Fec^+$: $y = 0.32x + 17.9$, $r = 0.95$ and 0.96 , respectively). In contrast, in antral follicles (> 200 µm in diameter), oocyte diameter was similar in the two genotypes ($Fec^B Fec^B$: 126.6 ± 3.2 , $Fec^+ Fec^+$: 122.6 ± 4.2 µm).

Experiment 2: protein patterns of small–large antral follicles

Forty-four oocytes from $Fec^B Fec^B$ and 33 from $Fec^+ Fec^+$ follicles were obtained after dissection. Of these, six $Fec^B Fec^B$ and four $Fec^+ Fec^+$ oocyte–cumulus complexes were incomplete and these oocytes were not considered further. The respective proportion of oocytes in the < 118 µm, 120–130 µm and > 130 µm diameter groups were 32, 45 and 22% for the $Fec^B Fec^B$ oocytes and 18, 54 and 27% for the $Fec^+ Fec^+$ oocytes.

There was no significant difference in the amounts of label incorporated in oocytes of either genotype ($Fec^B Fec^B$: $148\,000 \pm 18\,000$ d.p.m. versus $Fec^+ Fec^+$: $135\,000 \pm 17\,000$ d.p.m.). In addition, incorporation was not related to oocyte size in either genotype ($r = 0.14$, $n = 67$). Representative patterns of the proteins neosynthesized by the oocytes after 3 h culture with [³⁵S]methionine are shown (Fig. 2). Forty-six protein bands, with molecular weights ranging from 20 to 100 kDa could be identified on the master lane summarizing all bands present.

Fig. 2. Patterns after one-dimensional PAGE of proteins neosynthesized (^{35}S incorporation) by representative oocytes from five $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ and five $\text{Fec}^{+}\text{Fec}^{+}$ ewes. Molecular weight markers are indicated on the left. Bands for which effects are described in the text are shown as (◄) for quantitative effects, as (◄◄) for size effects and as (◄) for genotype $\times$ size interaction.

After image analysis, no qualitative difference related to genotype or to oocyte size could be identified. In contrast, the two way ANOVA on the amounts of proteins contained in the 23 most frequent bands demonstrated a number of differences related to genotype, oocyte size or their interaction. Four bands (B15, B20, B32 and B43) at 74, 59, 37 and 25 kDa, respectively, displayed quantitative differences among genotypes. Amounts of proteins contained in bands 20 and 32 were significantly increased in oocytes of $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ ewes (band 20, $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$: 4580 ± 800 darkness units versus $\text{Fec}^{+}\text{Fec}^{+}$: 1690 ± 500 darkness units, $P = 0.01$; band 32, $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$: $11\,120 \pm 1700$ darkness units versus $\text{Fec}^{+}\text{Fec}^{+}$: 6340 ± 1000 darkness units, $P = 0.02$).

In contrast, amounts of proteins contained in bands 15 and 43 were significantly higher in $\text{Fec}^{+}\text{Fec}^{+}$ ewes (band 15, $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$: 3550 ± 800 darkness units versus $\text{Fec}^{+}\text{Fec}^{+}$: 7700 ± 1400 darkness units, $P = 0.02$; band 43, $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$: 5000 ± 1160 darkness units versus $\text{Fec}^{+}\text{Fec}^{+}$: $10\,300 \pm 1800$ darkness units, $P = 0.02$).

Oocyte diameter had a limited effect on the pattern of neosynthesized proteins. A single band (band 37) at 30 kDa

was significantly altered by oocyte size (diameter $< 118\ \mu\text{m}$: 5890 ± 1260 darkness units; $120\text{--}130\ \mu\text{m}$: 3900 ± 520 darkness units; $> 130\ \mu\text{m}$: 7150 ± 1530 darkness units).

For two protein bands (band 25 and band 26) at 45 kDa and 43 kDa, respectively, a significant interaction between genotype and size was detected (band 25, $P = 0.06$; band 26, $P < 0.01$). For band 25, a steady increase in the amounts of this protein was associated with increased oocyte size in $\text{Fec}^{+}\text{Fec}^{+}$ oocytes while an opposite trend was present in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ oocytes (Fig. 3a). For band 26, amounts of proteins in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ oocytes were low and slightly decreased with oocyte size, while in $\text{Fec}^{+}\text{Fec}^{+}$ oocytes, their amounts increased markedly in the largest oocytes (Fig. 3b).

The amounts of 17α -hydroxylase present in the follicular wall of individual follicles were unaffected by breed ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$: $25\,000 \pm 5700$ darkness units versus $\text{Fec}^{+}\text{Fec}^{+}$: $22\,800 \pm 4700$ darkness units) and by follicle size ($r = 0.22$ and 0.31 in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ and $\text{Fec}^{+}\text{Fec}^{+}$ follicles, respectively). Amounts of this enzyme per mg of tissue were highly variable among follicles. Because the distribution of these amounts was bimodal, a threshold of

Fig. 3. Interactions between genotype and follicle size for the amounts of proteins contained in (a) band 25 and (b) band 26 in oocytes belonging to follicles from $Fec^B Fec^+$ (■) and $Fec^+ Fec^+$ (□) ewes.

10 000 darkness units could be established. Making the assumption that low amounts of 17 α -hydroxylase were associated with follicular atresia (Huet *et al.*, 1997), 50% and 44% of the $Fec^B Fec^B$ and $Fec^+ Fec^+$ follicles were in advanced atresia. None of the bands identified as variable with genotype and size (bands 15, 20, 25, 26, 32, 37 and 43) was affected by atresia of the follicle. Furthermore, the amounts of protein present in each band could not be related to the amounts of 17 α -hydroxylase present in the follicular wall. This suggests that follicle atresia has minimal effects on the pattern of proteins neosynthesized by the oocytes.

Experiment 3: nuclear maturation of oocytes from follicles of different sizes

All the oocytes fixed at $t = 0$ were at the GV stage. No significant difference was observed between the size of oocytes isolated from $Fec^B Fec^+$ and $Fec^+ Fec^+$ ewes and among oocytes from different follicular size classes (Table 1). The meiotic stages reached after 24 h culture of cumulus–oocytes complexes were evaluated for both genotypes (Table 2). Successful GVBD and progression to metaphase II occurred in 35% of $Fec^B Fec^+$ and 11% of $Fec^+ Fec^+$ ($P < 0.02$) oocytes from small follicles (0.6–1.0 mm). However, in this class of follicle, cumulus expansion was complete only in $Fec^B Fec^+$, while only partial in $Fec^+ Fec^+$ after 24 h culture. In $Fec^B Fec^+$ ewes, 70% of oocytes from 1.1–1.5 mm follicles were meiotically competent, in contrast to oocytes from the same class of follicles in $Fec^+ Fec^+$ ewes, where only 47% reached metaphase II ($P < 0.05$). Even in

Table 1. Effect of follicular size and genotype on oocyte diameter in ewes with or without the Booroola gene (Expt 3)

Follicle size (mm)	Genotype	Number of oocytes examined	Oocyte diameter (μ m) $\pm$ SEM
0.6–1.0	$Fec^+ Fec^+$	4	136 $\pm$ 9.9
	$Fec^B Fec^+$	12	132 $\pm$ 8.8
1.1–2.0	$Fec^+ Fec^+$	12	136 $\pm$ 4.3
	$Fec^B Fec^+$	32	139 $\pm$ 8.2
2.1–3.5	$Fec^+ Fec^+$	18	143 $\pm$ 13.2
	$Fec^B Fec^+$	46	139 $\pm$ 11.3
> 3.5	$Fec^+ Fec^+$	8	143 $\pm$ 8.2
	$Fec^B Fec^+$	14	136 $\pm$ 2.7

follicles > 1.6 mm in diameter, 98% of oocytes from $Fec^B Fec^+$ follicles reached metaphase II, while a significant proportion (16%) of $Fec^+ Fec^+$ follicles yielded oocytes that were blocked in metaphase I (Table 2).

Experiment 4: effects of FSH on the ability of the oocytes to develop to the blastocyst stage after maturation and fertilization in vitro

The effect of addition of FSH to the maturation medium containing 10% follicular fluid on meiotic maturation is shown (Table 3). In both genotypes, the oocyte MII competence was greatly increased by FSH ($P < 0.002$ for $Fec^+ Fec^+$ and $P < 0.001$ for $Fec^B Fec^+$) and $Fec^B Fec^+$ oocytes had a higher maturation rate than non-carrier oocytes in the presence (90% versus 78%, $P < 0.01$) but not in the absence (46% versus 56%, $P > 0.05$) of FSH in the medium. In $Fec^B Fec^+$ oocytes, the maturation rate was two fold higher ($P < 0.001$) in the presence of FSH, for oocytes from small and medium sized follicles compared with only a 30% increase ($P > 0.05$) in the same follicular class of non-carrier oocytes. In addition, in the absence of FSH, 10% of $Fec^B Fec^+$ versus 1% of $Fec^+ Fec^+$ oocytes ($P < 0.02$) were blocked at the GV stage.

Since immature oocytes were not eliminated after IVM owing to the difficulty of visualizing the polar body in matured eggs, the cleavage rates reflected the maturation rates (Table 4). In the absence of FSH, the cleavage rate was not as significantly depressed in $Fec^+ Fec^+$ (60 versus 70%) as it was in $Fec^B Fec^+$ oocytes (48% versus 77%, $P < 0.001$).

The developmental rate of cleaved eggs was affected ($P < 0.01$) by follicular size in both genotypes (Table 4). $Fec^B Fec^+$ oocytes had a greater ability to develop than $Fec^+ Fec^+$ oocytes in the class of small follicles ($P < 0.05$) but the difference was not significant for the oocytes from the larger classes. The ability of $Fec^B Fec^+$ oocytes to develop was similar to that of the upper class of $Fec^+ Fec^+$ oocytes. Omitting FSH from the IVM medium did not significantly reduce the percentage of cleaved eggs developing to the blastocyst stage: 15% versus 23% and 27% versus 36%, respectively, in $Fec^+ Fec^+$ and $Fec^B Fec^+$ eggs. However, a significant difference ($P < 0.05$) was found when the two genotypes were pooled, since, in the presence of FSH, 30% of the fertilized oocytes developed to the blastocyst stage, while this proportion dropped to 20% in the absence of FSH during

Table 2. Influence of follicle size on *in vitro* maturation of oocytes from ewes with or without the Booroola gene (Expt 3)

Follicle size (mm)	Genotype	Number of oocytes	Meiotic stages reached after 24 h (% of oocytes)			
			GV	Prometaphase I	Metaphase I	Metaphase II
0.6–1.0	<i>Fec⁺Fec⁺</i>	37	32	30	27	11
	<i>Fec^BFec⁺</i>	48	25	10	29	35
1.1–1.5	<i>Fec⁺Fec⁺</i>	36	3	28	22	47
	<i>Fec^BFec⁺</i>	63	—	14	16	70
≥ 1.6	<i>Fec⁺Fec⁺</i>	79	—	1	16	82
	<i>Fec^BFec⁺</i>	61	—	—	2	98

Cumulus cell-enclosed oocytes were cultured for 24 h in medium containing 10% sheep follicular fluid and 100 ng FSH ml⁻¹. GV: germinal vesicle.

Table 3. Influence of FSH (100 ng ml⁻¹) on *in vitro* maturation of oocytes from ewes in the absence (*Fec⁺Fec⁺*) or presence (*Fec^BFec⁺*) of the *Fec^B* gene (Expt 4)

Follicle size (mm)	Oocyte matured with (+) or without (–) FSH	<i>Fec⁺Fec⁺</i>		<i>Fec^BFec⁺</i>	
		Number of oocytes	Percentage at metaphase II	Number of oocytes	Percentage at metaphase II
1–2	+	35	71	45	84
	–	30	47	56	36
2.0–3.5	+	40	75	40	90
	–	44	57	45	49
> 3.5	+	16	100	30	100
	–	14	71	24	79
Total	+	91	78	115	90
	–	88	56	125	49

maturation. The hatching rate at day 7 was unaffected by genotype (*Fec^BFec⁺*: 42% versus *Fec⁺Fec⁺*: 50%) and the presence of FSH (FSH: 45% versus no FSH: 43%).

Discussion

The main conclusions of this study are: (1) the ability of the ovine oocyte to resume meiosis and to gain the ability to develop to the blastocyst stage is acquired sequentially as the follicle enlarges but earlier in *Fec^B* gene carriers than in non-carriers. (2) The pattern of oocyte growth in preantral follicles is altered in homozygous carriers of the *Fec^B* gene as these oocytes are larger than those from *Fec⁺Fec⁺* ewes for specific follicle sizes. (3) Quantitative differences in the protein patterns of oocytes specific to follicle size and genotype were identified. (4) Qualitative differences specific to genotype were not found when oocytes from *Fec^BFec^B* and *Fec⁺Fec⁺* ewes were compared.

Oocytes in most mammals are arrested at the dictyate stage of prophase I at the end of oogenesis. They gain the ability to resume meiosis when explanted from the follicle once they have reached a specific size (Eppig, 1993). Fuhrer *et al.* (1989) have shown that only 1.5% of bovine oocytes from follicles < 0.9 mm in diameter had the ability to reach metaphase II. Motlik *et al.* (1984) reported that only 20% of pig oocytes < 0.7 mm in diameter could resume meiosis after 24 h culture. Similarly, recent data in goats (De Smedt *et al.*, 1994) established that oocytes acquire the ability to reach metaphase I in follicles 1–2 mm and to reach metaphase II in follicles > 3 mm in diameter. The present data extend these conclusions to sheep. Expt 3 demonstrated in non-carriers that very few oocytes (11%) from follicles < 1 mm in diameter had the ability to reach metaphase II. This proportion increased to 47% and 82%, respectively, for follicles 1.0–1.5 mm and > 1.6 mm in diameter. The ability to resume meiosis was acquired earlier in follicles of *Fec^B* gene carrier ewes. The ability of oocytes from small follicles (0.6–1.0 mm in diameter) of *Fec^BFec⁺* ewes

Table 4. Effect of follicle size and the presence of FSH during maturation on cleavage and development *in vitro* of fertilized oocytes from ewes with or without the Booroola gene after 7 days of culture in synthetic oviductal fluid medium

Follicle size (mm)	Oocyte matured with (+) or without (-) FSH	Non-carriers			Carriers		
		Number of oocytes	Percentage of cleaved eggs	Percentage of blastocysts	Number of oocytes	Percentage of cleaved eggs	Percentage of blastocysts
1–2	+	60	65	13	103	73	28
	–	63	59	8	91	47	16
2.0–3.5	+	72	81	26	46	78	44
	–	69	65	20	50	42	38
> 3.5	+	24	63	40	26	88	48
	–	23	52	17	22	64	43
Total	+	161	70	23 ^a	175	77	36 ^b
	–		n.s.			(<i>P</i> < 0.001)	
		155	60	15	163	48	27

^a and ^b are not significantly different. ^a + ^b*P* < 0.05 (it is only when both genotypes are combined that the FSH effect becomes significant).

to resume meiosis was threefold higher than those of *Fec*⁺ ewes. A higher proportion of oocytes of *Fec*^B*Fec*⁺ ewes originating from follicles 1.0–1.5 and > 1.6 mm in diameter to resume meiosis was also observed. These differences could only be visualized when the oocytes were matured in the presence of FSH (100 ng ml⁻¹). Whether this is the result of a difference related to genotype in the appearance or development of FSH receptors on their cumulus cells remains to be investigated.

When cytoplasmic maturation (that is, the ability of the oocyte to develop to the blastocyst stage after IVM/IVF) was considered, it was also found that lower rates of embryonic development were obtained with oocytes coming from small follicles. For example, in the non-carrier group treated with FSH, the percentage of blastocysts increased from 13 to 40% when oocytes were obtained in follicles 1–2 mm or > 3 mm in diameter, respectively. Such an effect has already been documented in cattle (Pavlok *et al.*, 1992, Lonergan *et al.*, 1994) and goats (Crozet *et al.*, 1995). As for completion of nuclear maturation (that is, the percentage of oocytes in metaphase II after 24 h culture), oocytes originating from *Fec*^B*Fec*⁺ follicles had greater ability to develop to the blastocyst stage in smaller follicles (28% and 13% for follicles 1–2 mm in diameter, 44% and 26% for follicles 2.0–3.5 mm in diameter in *Fec*^B*Fec*⁺ and *Fec*⁺*Fec*⁺, respectively). This ability was again more obvious in the presence of FSH (100 ng ml⁻¹). The higher requirement for FSH of oocytes of *Fec*^B*Fec*⁺ follicles, as visualized by the proportion of oocytes reaching metaphase II and the proportion of oocytes cleaved is in good agreement with the high circulating FSH concentrations in *Fec*^B*Fec*⁺ ewes (McNatty *et al.*, 1987).

The mechanisms whereby the oocytes in *Fec*^B gene carriers gain the ability to mature earlier are unknown. In Expt 1, the pattern of growth of the oocytes of *Fec*^B*Fec*^B versus *Fec*⁺*Fec*⁺ preantral follicles was compared. While the general pattern of oocyte growth was similar to that described by Cahill and Mauleon (1980), a clear difference related to the *Fec*^B gene was observed. Oocytes in primordial follicles were of similar size in *Fec*^B*Fec*^B and *Fec*⁺*Fec*⁺ follicles. However, when the follicles were between 50 and 200 µm in diameter, the *Fec*^B*Fec*^B oocytes

were always larger for a specific follicle size than those of *Fec*⁺*Fec*⁺ follicles. This indicates that, already in preantral follicles, the relationships between the oocyte and its somatic cells are altered. That the function of preantral follicles is altered by the *Fec*^B gene has already been demonstrated using mitotic index of the granulosa cells as a marker (Driancourt *et al.*, 1985). Whether there is a relationship between this altered oocyte growth in *Fec*^B*Fec*^B follicles undergoing preantral development and the earlier ability of the *Fec*^B*Fec*^B oocytes to undergo nuclear and cytoplasmic maturation remains to be explored.

These differences in oocyte growth and maturation related to the *Fec*^B gene were investigated by examining the proteins produced by the oocyte itself. Expt 2 used one-dimensional SDS-PAGE on proteins neosynthesized by individual oocytes. Forty-six bands could be visualized between 100 and 20 kDa, a number close to that reported earlier in ovine oocytes by Crosby *et al.* (1981). As in this earlier study, major proteins were identified at 45 and 30 kDa. However, the present study is the first to report (1) oocyte size effects on the amounts of specific oocyte proteins in sheep, and (2) *Fec*^B gene effects on these proteins. Oocyte size significantly altered the amounts of a 30 kDa protein. A 30 kDa protein was also affected by follicle size in mouse oocytes (Schultz and Wassarman, 1977). A genotype by size interaction was identified for two other bands at 45 and 43 kDa. Band 25 at 45 kDa increased with size in oocytes of *Fec*⁺*Fec*⁺ oocytes, while it decreased in *Fec*^B*Fec*^B oocytes. Band 26 at 43 kDa increased with size in oocytes of *Fec*⁺*Fec*⁺ oocytes only. In any case, none of the bands affected by oocyte size in the present study (45, 43 and 30 kDa) had a molecular weight approaching those of p34 cdc 2 and cyclin B1 (34 and 65 kDa respectively), the two components of maturation promoting factor (MPF) (Fulka, 1985). The Booroola genotype affected the amounts of proteins contained in four bands at 74, 59, 37 and 25 kDa with greater amounts of proteins for *Fec*^B*Fec*^B oocytes at 59 and 37 kDa and the converse for the two other bands at 74 and 25 kDa. The nature of these proteins and their role in oocyte growth and differentiation remain to be studied.

The present study could not demonstrate qualitative differences between $Fec^B Fec^B$ and $Fec^+ Fec^+$ oocytes contained in antral follicles. This contrasts with earlier findings using two-dimensional electrophoresis on somatic cells which showed that two protein spots in proteins neosynthesized by intact follicles are qualitatively affected by the Fec^B gene (Driancourt et al., 1996). Further studies of the pattern of protein synthesis by oocytes from preantral follicles are required to check whether qualitative differences can be identified at this stage.

In conclusion, all four experiments demonstrated that the Fec^B gene, in addition to its effects on granulosa cells (Montgomery et al., 1992), also affects oocyte development and function.

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