Oocytes in sheep homozygous for a mutation in bone morphogenetic protein receptor 1B express lower mRNA levels of bone morphogenetic protein 15 but not growth differentiation factor 9

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Abstract

The aim of this study was to test the hypothesis that the high ovulation rate in ewes (BB) homozygous for a mutation in the bone morphogenetic protein receptor type 1B (*BMPR1B*) gene is linked to lower *BMP15* and/or *GDF9* mRNA in oocytes compared with those in wild-type (++) ewes. Cumulus cell–oocyte complexes (COC) and granulosa cells (GC) were recovered from ≥ 1 mm diameter follicles of BB and ++ ewes during a prostaglandin-induced follicular phase. Expression levels of *GDF9* and *BMP15* were measured by multiplex qPCR from individual COC. The gonadotropin-induced cAMP responses of the GC from each non-atretic follicle were measured following treatment with FSH or human chorionic gonadotropin. In a separate validation experiment, *GDF9* and *BMP15* expression was present only in oocytes and not in cumulus cells. There was no effect of follicular diameter on oocyte-derived *GDF9* or *BMP15* mRNA levels. The mean expression levels of *BMP15*, but not *GDF9*, were significantly lower in all non-atretic follicles, including the subsets containing either FSH- or LH-responsive GC in BB, compared with ++, ewes. No genotype effects were noted for FSH-induced cAMP production by GC either with respect to dose of, or number of follicles responding to, FSH. However, ovaries from BB ewes contained significantly more follicles responsive to LH, with respect to cAMP production in GC. We propose that these findings are consistent with the hypothesis that the higher ovulation rate in BB sheep is due, at least in part, to lower oocyte-derived *BMP15* mRNA levels together with the earlier onset of LH-responsiveness in GC.

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Introduction

Ewes that inherit a mutation in the bone morphogenetic protein receptor type 1B (*BMPR1B*; also known as activin-like kinase 6) gene have significantly higher ovulation rates compared with their wild-type contemporaries (see reviews by McNatty *et al.* (2005) and Fabre *et al.* (2006)). These *BMPR1B*-mutant ewes are commonly referred to as Booroola ewes. It has been proposed that the Booroola mutation influences the level of oocyte-derived signalling, possibly via altered protein expression of BMP15 (McNatty *et al.* 2005) or via altered BMP15 signalling (Mulsant *et al.* 2001, Campbell *et al.* 2006). However to our knowledge, this has not been proven. In ewes heterozygous for an inactivating mutation in BMP15, such as the Inverdale (FecX^I) or Hanna (FecX^H) genotype, it would be reasonable to expect lower levels of oocyte-secreted protein for BMP15 (Galloway *et al.* 2000). A physiological relationship between lower

biologically active concentrations of BMP15 and higher ovulation rates has been demonstrated by immunising wild-type ewes against the entire mature BMP15 protein or small peptide sequences of the mature BMP15 region (Juengel *et al.* 2002, McNatty *et al.* 2007). Collectively, the evidence from both the immunisation and mutation studies indicates that as the dose of biologically available BMP15 decreases, the ovulation rate increases until BMP15 concentrations are too low to support follicular growth (McNatty *et al.* 2004, 2007, Fabre *et al.* 2006). At this point, animals become infertile, as is the case in homozygous BMP15-mutant animals (Galloway *et al.* 2000, Scaramuzzi *et al.* 2011). The notion that altered BMP15 synthesis is in some way linked to the *BMPR1B* mutation is consistent with the observation that ewes with both an inactivating *BMP15* mutation and *BMPR1B* mutation have ovulation rates that are more than additive (Davis *et al.* 1999, McNatty *et al.* 2005).

Recently, the higher ovulation rates in Inverdale ewes were found to be associated with an earlier acquisition of LH (but not FSH) responsiveness by granulosa cells (GC) in a greater proportion of developing antral follicles (McNatty *et al.* 2009). In homozygous mutant Booroola (BB) ewes, ovarian follicles ovulate at smaller diameters (i.e. >2.5–4.5 mm) compared with those in the wild-type ewes (i.e. >4.5 mm), with the ovulating follicles in BB ewes each containing fewer GC (McNatty *et al.* 1986, Henderson *et al.* 1987). Moreover, similar to that which occurs in Inverdale ewes, GC in BB ewes develop a responsiveness to LH earlier in follicular development, consistent with the reduced size of the ovulating follicles (Henderson *et al.* 1987). It should be noted that although the GC in small (i.e. 1–2.5 mm) antral follicles of BB ewes also showed an increased sensitivity to FSH with respect to cAMP production (Henderson *et al.* 1987), the FSH preparations used in this experiment contained >1% contamination of LH (McNatty *et al.* 2009).

The aim of this study was to test the hypothesis that the increased ovulation rate in BB ewes, compared with those in wild-type (+ +) ewes, is linked, at least in part, to lower the mRNA expression levels of *BMP15* and/or *GDF9* in oocytes of follicles, particularly in those containing GC that are responsive to LH with regard to cAMP production.

Results

Effect of genotype on ovulation rate

The mean (and range of) ovulation rates based on the number of corpora lutea (CL) present in the wild-type (+ +) and homozygous mutant (BB) ewes were 2.8 (2–5) and 9.7 (8–11) respectively.

Effect of genotype on number of GC with respect to follicular diameter

With respect to the number of GC present within follicles, there were significant main effects of follicular diameter ($P < 0.001$) and genotype ($P < 0.001$), but had no interactions (Fig. 1).

Effects of dose, follicular diameter and genotype on FSH- or LH-induced cAMP responses in GC

Follicles described as FSH- or LH-responsive contained GC that resulted in the production of cAMP of >10 pmol/ 10^6 cells after treatment with either FSH or LH respectively. Those follicles that were defined as LH-responsive also responded to FSH in 11/11 follicles from + + ewes and 25/26 follicles from BB ewes. The GC from the one (1/26) follicle from a BB ewe that was defined as LH-responsive, but not FSH-responsive, elicited a cAMP response of 7 pmol/ 10^6 cells after treatment with 1000 ng/ml of FSH.

Figure 1 Effect of genotype and follicular diameter on number of granulosa cells (GC) present in non-atretic follicles. The numbers of GC are represented by black bars for wild-type (+ +) ewes and white bars for homozygous Booroola mutant (BB) ewes. Values are expressed as geometric means and error bars denote 95% confidence limits. Numbers of follicles are shown in parentheses.

The effects of FSH dose, follicular diameter and genotype on FSH-induced cAMP response of GC are shown in Fig. 2. There were highly significant effects of dose (10, 100 and 1000 ng/ml; $P < 0.001$) and follicular diameter (1–2.5 and >2.5 mm; $P < 0.001$), but not genotype (Fig. 2), on FSH-induced cAMP production in GC and no interactions were observed. The proportion of non-atretic follicles containing FSH-responsive GC was similar ($P > 0.05$) between + + (36/48; 75%) and BB (34/50; 68%) ewes.

There were significant effects of follicular diameter ($P < 0.01$) and genotype ($P < 0.05$) on LH-induced cAMP production in GC, but no interactions were observed. The geometric mean (and 95% confidence limits) levels for the LH-induced cAMP levels for 1–2.5 and >2.5–4.5 mm diameter follicles in BB ewes were 4 (2, 10) and 16 (6, 46) pmol/ 10^6 GC, respectively (no follicles >4.5 mm diameter were present). For the + + ewes, the cAMP values were 0.8 (0.4, 1.4), 3.2 (0.6, 17.0) and 29 (6, 132) pmol/ 10^6 GC for the 1–2.5, >2.5–4.5 and >4.5 mm diameter follicles respectively. The proportion of non-atretic follicles containing LH-responsive GC was higher ($P < 0.001$) in BB (26/50; 52%), compared with + + (11/48; 23%) ewes. This difference in the proportion of LH-responsive follicles was due to responsiveness being attained in more smaller-sized follicles in BB ewes, compared with + + ewes. Specifically, 41% of small (1–2.5 mm) and 54% of large (>2.5 mm) follicles were LH-responsive in BB ewes compared with only 16 and 44%, respectively, of those in + + ewes.

Specificity testing of *GDF9* and *BMP15* in dissociated cumulus cells and denuded oocytes

The localisation of mRNA expression of *GDF9* and *BMP15*, together with gap junction protein alpha 4 (*GJA4*, also known as connexin 37 (CX37) a specific marker of oocytes), *GJA1* (CX43) and *FSHR* (specific

Figure 2 Effect of follicular diameter and dose of FSH on cAMP production by granulosa cells from follicles of wild-type (++; black bars) and homozygous Booroola mutant (BB; white bars) ewes combined. Follicles 1–2.5 mm in diameter are represented in the top graph and >2.5 mm diameter are represented in the bottom graph. Values are expressed as geometric means and error bars denote 95% confidence limits. The number of observations across the doses for the 1–2.5 mm follicles is between 39 and 58 and for those >2.5 mm is between 31 and 33.

markers of granulosa and cumulus cells), within ovine cumulus cell-oocyte complexes (COC) is shown in Fig. 3. Expression of *GDF9*, *BMP15* and *GJA4* mRNA was present only in oocytes, whereas expression of *GJA1* and *FSHR* mRNA was present only in the cumulus cells.

Effect of follicular health, genotype and gonadotropin-responsiveness of GC on relative expression levels of *GDF9* and *BMP15* mRNA in oocytes

These data are summarised in Table 1. The mRNA values for *GDF9* and *BMP15* with respect to follicular health, genotype and gonadotropin-responsiveness of GC were corrected against a control (calibrator) sample containing differing amounts of *GDF9* and *BMP15* mRNA, and are referred to herein as relative mRNA levels. Hence, comparisons of the mRNA expression levels of *GDF9* within and between genotypes and of *BMP15* within and between genotypes were made with relative mRNA expression levels. Also given in Table 1 are the ratios for *GDF9/BMP15* mRNA with respect to follicular health, genotype and gonadotropin-responsiveness of GC. These ratio values were obtained by comparing cycle threshold (Ct) values, and were not corrected against the calibrator sample.

There was no significant effect of follicular diameter on the mean relative expression levels of either *GDF9* or *BMP15* mRNA (data not shown). For both genes, relative expression levels were higher in non-atretic versus atretic follicles. The mean relative *GDF9* mRNA expression levels were similar between genotypes. For the follicles containing GC that were responsive to LH, the relative *GDF9* mRNA levels in BB, compared with ++, ewes tended to be lower ($P=0.062$) but was not significantly different. Conversely, with respect to relative *BMP15* expression levels, a significant effect of genotype was noted for oocytes from non-atretic follicles and from the subsets of FSH- and LH-responsive follicles. In all instances, the relative *BMP15* mRNA levels were lower in oocytes from the BB ewes ($P<0.05$). The mean ratios of *GDF9:BMP15* irrespective of follicular health for the ++ and BB genotypes were 1.19 ± 0.04 and 1.26 ± 0.04 respectively. These tended to be higher in the BB ewes ($P=0.05$), with a greater number ($P<0.05$) of COC with a *GDF9:BMP15* ratio of >1.1 in BB compared with ++ ewes. However, no genotype differences were noted for *GDF9:BMP15* ratios when follicles were further classified into healthy, atretic or with FSH- or LH-responsive GC (Table 1).

The cycle threshold (Ct) values indicating the expression levels of *GDF9* and *BMP15* within individual oocytes of ++ and BB ewes were highly correlated (Fig. 4; ++, $R^2=0.9362$, $P<0.0001$; BB, $R^2=0.9364$, $P<0.0001$).

Discussion

The key findings from this study were that *GDF9* and *BMP15* mRNA are expressed exclusively by oocytes in the COC; *GDF9* and *BMP15* mRNA levels are strongly correlated in both genotypes; and *BMP15*, but

Gene	Cumulus cells	Denuded oocyte
<i>GDF9</i>	0	1.00 (0.74–1.34)
<i>BMP15</i>	0	1.04 (0.69–1.59)
<i>GJA4</i>	0	0.53 (0.38–0.75)
<i>GJA1</i>	0.11 (0.07–0.16)	0
<i>FSHR</i>	0.08 (0.05–0.13)	0

Figure 3 Relative gene expression levels of *GDF9*, *BMP15*, *GJA4*, *GJA1* and *FSHR* in cumulus cells and denuded oocytes from individual cumulus cell-oocyte complexes ($n=6$). Values are corrected for *RPL19* (housekeeping gene) and expressed as means (and errors) relative to the mean value of oocyte-derived *GDF9* mRNA expression.

Table 1 Relative expression levels of *GDF9* and *BMP15* mRNA, and *GDF9/BMP15* ratios of cumulus cell–oocyte complexes, with respect to genotype, follicular health and cAMP responsiveness of granulosa cells (GC) to either FSH or LH, from homozygous Booroola mutant (BB) and wild-type (+ +) ewes.

Follicular type	GDF9		BMP15		GDF9/BMP15 ratios	
	++	BB	++	BB	++	BB
Non-atretic	0.72 ± 0.06 ^a	0.66 ± 0.06 ^a	0.91 ± 0.07 ^a	0.67 ± 0.06 ^b	1.19 ± 0.05 ^a n=56	1.22 ± 0.05 ^a n=73
Atretic	0.42 ± 0.06 ^{a,*}	0.42 ± 0.06 ^{a,*}	0.52 ± 0.09 ^{a,*}	0.49 ± 0.10 ^{a,*}	1.14 ± 0.06 ^a n=26	1.23 ± 0.04 ^a n=30
With FSH-responsive GC	0.78 ± 0.06 ^a	0.72 ± 0.06 ^a	0.99 ± 0.08 ^a	0.63 ± 0.07 ^b	1.10 ± 0.03 ^a n=36	1.35 ± 0.13 ^a n=30
With LH-responsive GC	1.02 ± 0.12 ^a	0.66 ± 0.12 ^a	1.06 ± 0.17 ^a	0.67 ± 0.09 ^b	1.24 ± 0.08 ^a n=7	1.20 ± 0.04 ^a n=23

Values are expressed as means ± S.E.M. The number of observations for each genotype is indicated in the ratio column. For each variable (*GDF9*, *BMP15* or *GDF9/BMP15* ratio), values in each row with different alphabetical superscripts are significantly different ($P < 0.05$) from one another. Asterisks indicate that the values for atretic follicles are significantly ($P < 0.05$) lower than those for the non-atretic follicles for each genotype.

not *GDF9*, mRNA levels in oocytes of BB ewes were lower than that in oocytes of ++ ewes. The finding from our QPCR studies that *GDF9* and *BMP15* mRNA are expressed only in oocytes supports previously published *in situ* hybridisation (ISH) and immunohistochemistry data (Galloway *et al.* 2000, McNatty *et al.* 2005, 2006). Recent studies in mice, pigs and humans have suggested that *BMP15* and *GDF9* are present in granulosa and/or cumulus cells as well as in oocytes of large antral follicles (Margoulis *et al.* 2009, Sun *et al.* 2010). It could be argued that ISH is not sufficiently sensitive to detect the low expression levels of *BMP15* or *GDF9* mRNA in granulosa or cumulus cells. However, the evidence from this study shows clearly that *GDF9* and *BMP15* mRNA were expressed exclusively in oocytes, and not in surrounding somatic cells devoid of oocyte contamination, of antral follicles at various developmental stages in sheep. The highly correlative relationship between mRNA expression levels of *GDF9* and *BMP15*, regardless of genotype, suggests that the regulation of these genes is very tightly regulated with expression levels higher in non-atretic than atretic follicles but with no effect of diameter of the antral follicles.

The evidence from this study that the *BMP15* mRNA levels were lower in BB compared with ++ ewes supports the notion that the oocyte is implicated in influencing the ovulation rate in these animals. It is possible that the *GDF9* mRNA levels in follicles with LH-responsive GC were also lower in BB than in wild-type ewes, but the sample size was not large enough for a statistical difference to be observed. It is known that ewes heterozygous for mutations in both *BMP15* and *GDF9* have ovulation rates that are greater than additive for each mutation alone (McNatty *et al.* 2005). Therefore, the possibility exists that both genes are expressed at lower levels and that both contribute to the high ovulation rates in these animals. However, until a significant effect for *GDF9* is established in a larger study, only lower expression levels of *BMP15* mRNA are able to be related to the higher ovulation rate in BB ewes.

Currently, seven different point mutations in the *BMP15* gene and three in the *GDF9* gene have been identified that influence ovulation rate in a number of different sheep breeds (see McNatty *et al.* (2005) and Paulini & Melo (2011) for reviews). Moreover, in sheep carrying the Woodlands FecX2^W mutation that also affects ovulation rate, the expression levels of *BMP15*, but not *GDF9*, mRNA are significantly lower than in the wild type. In FecX2^W animals, the mutation appears to be on the X-chromosome but has not been linked to any known mutation in the *BMP15* gene (Feary *et al.* 2007). However, as is the case with the BB ewes, a downstream consequence is a reduced expression level of *BMP15* mRNA (Feary *et al.* 2007). Moreover, the results raise the possibility that, at least in part, one common mechanism might exist between ewes with the *BMP15*, Woodlands and the *BMP1B* (Booroola) mutations in that all may have lower *BMP15* protein levels in the developing

Figure 4 Regression plots of cycle threshold (Ct) values and lines of best fit for *BMP15* and *GDF9* genes from individual cumulus cell–oocyte complexes from wild-type (++) (black circles; solid line) and homozygous Booroola mutant (BB; white circles; dashed line) ewes. Equations of lines of best fit and the coefficients of determination (R^2) values are presented for both sheep genotypes.

follicles. Of interest is that Inverdale and Booroola ewes have a greater proportion of follicles developing an earlier onset of LH-responsiveness and thereby ovulating at a smaller diameter (McNatty *et al.* 2009; this study). However, whether the earlier acquisition of LH-responsiveness is directly due to lower concentrations of oocyte-secreted BMP15 protein will require further studies including the measurement of this growth factor in follicular fluid. Currently, no assay for the accurate measurement of BMP15 in biological fluid is available. Owing to a lack of sensitivity, quantification of BMP15 by western blotting using specific BMP15 antibodies would require pooling of follicular fluid from many follicles. Our initial attempts to quantify BMP15 in ovine follicular fluid using a western blotting procedure have been unsuccessful despite the application of an affinity-enrichment step and a highly specific monoclonal antibody. A preferred alternative approach might be the application of a more sensitive two-site sandwich-based ELISA assay and a purified biologically active reference standard; however, this methodology is not yet available.

Although this study shows a decreased level of *BMP15* expression in oocytes of BB ewes, the link between this finding and the signalling pathway via the mutant *BMPR1B* remains obscure. Fabre *et al.* (2003) examined the effects of the Booroola genotype on *BMPR1B* signalling activity in HEK-293 cells transiently transfected with *BMPR2* and the wild type or mutant forms of *BMPR1B*. Their results showed an enhanced level of basal signalling activity but an absence of BMP4-stimulated signalling activity in the transfected mutant cells compared with wild-type cells. Campbell *et al.* (2006) reported that BMP2, 4 or 6 augmented the effects of FSH- and insulin-like growth factor 1 (IGF1)-induced oestradiol production by GC in BB compared with that in ++ ewes, indicating a direct link among BMPs, *BMPR1B* and follicular somatic cell maturation. However, at present, the specific ligand(s) that interacts with *BMPR1B* and the associated type II receptor are not known. Possible candidates include BMP15, BMP15–GDF9 heterodimeric complexes and BMP6 (McNatty *et al.* 2005, Juengel *et al.* 2006). It is unlikely that other BMPs such as 2, 4 and 7 are candidates as their expression levels in ovine follicular cells were undetectable by ISH (Juengel *et al.* 2006). Therefore, it is possible that the downstream consequences of the mutated *BMPR1B* in BB ewes are mediated solely via lower levels of expression of *BMP15* mRNA, leading to lower concentrations of BMP15 protein within follicular fluid although additional ligand-induced signalling effects via BMPs other than BMP15 cannot be ruled out.

This study showed that non-atretic, antral follicles, in comparison with atretic follicles, expressed higher levels of *BMP15* and *GDF9* mRNA. A reduction in mRNA levels of these growth factors in atretic follicles is likely to be due to the reduced support to the oocyte through the loss of cell-to-cell contacts between the GC, and

between the GC and the oocyte (Wiesen & Midgley 1994, Lenhart *et al.* 1998, Carabatsos *et al.* 2000). The cAMP responses induced by FSH were influenced by follicular diameter and dose of FSH, which is consistent with our earlier observations (Henderson *et al.* 1987). Previously, we also reported a genotype difference in the sensitivity of GC to FSH as well as LH (Henderson *et al.* 1987). However, we now know that the FSH preparation was contaminated with ~1–2% LH (McNatty *et al.* 2009). In this study, the highly purified FSH preparation contained no measurable LH (i.e. <0.002%) and there was no genotype effect of FSH on cAMP production. Thus, as with heterozygous *BMP15*-mutant Inverdale ewes (McNatty *et al.* 2009), the early maturation of antral follicles observed in BB ewes is unlikely to be due to a greater sensitivity to FSH, as known previously (Henderson *et al.* 1987).

Despite the genotype difference in levels of *BMP15*, but not *GDF9*, mRNA, the mean ratios of *GDF9*:*BMP15* mRNA in individual oocytes were not different between the genotypes, although the frequency of higher ratios was significantly lower in BB ewes. This is supported by a less positive slope of regression when Ct values for *BMP15* and *GDF9* in individual oocytes were plotted on the y and x axes, respectively, compared with that of ++ ewes. The lack of significant difference in the ratio values due to genotype is probably due to a combination of only a small difference between genotypes and substantial variability within individual oocytes. Despite this variability, the expression levels of *GDF9* compared with *BMP15* were similar (~1.2) irrespective of follicular diameter, health or gonadotropin-responsiveness. Earlier studies on assessing the production of BMP15 and GDF9 proteins following co-transfection of 293T cells indicate complex intracellular interactions between BMP15 and GDF9, indicating that the processing and secretion of these genes are not independent (see review by Moore & Shimasaki (2005)). This study clearly demonstrates that their expression levels are also unlikely to be independent of one another. Thus, the expression ratios of *GDF9*:*BMP15* are likely to have significant effects on downstream processing and in BMP15 and GDF9 protein levels. It is likely that the expression ratios of *GDF9*:*BMP15* mRNA will vary between mono- and poly-ovulatory species. For example, the rodent is thought to have little or no BMP15 protein expressed throughout the majority of follicular development (Hashimoto *et al.* 2005). Our preliminary evidence suggests that, indeed, the expression ratios of GDF9 and BMP15 vary between multiple and low ovulation rate species (Crawford & McNatty 2011).

Significant effects of genotype and follicular diameter were noted for human chorionic gonadotropin (hCG)/LH-responsive follicles, consistent with our earlier report (Henderson *et al.* 1987). That is, a much larger proportion of follicles ≥ 1 mm diameter responded to LH in BB, compared with ++, ewes. This increase in the

proportion of LH-responsive follicles in BB ewes was due mainly to more follicles of a smaller (1–2.5 mm) size attaining LH-responsiveness. In this study, the number of LH-responsive follicles was 11 ($N=4$) in ++, and 26 ($N=3$) in BB, ewes. If all these follicles went on to ovulate, this would correspond to ovulation rates of 2.75 and 8.7 in ++ and BB ewes respectively. The numbers of CL observed from the previous ovulatory cycle were 2.8 and 9.7 for ++ and BB ewes respectively. Interestingly, one of the ++ ewes contained five CL, which is rare for wild-type Romney ewes. However, the ovaries from these ewes contained follicles that were typical of those in ++ ewes, i.e. characteristically larger follicles with more GC. Importantly, oocytes recovered from LH-responsive follicles of BB, compared with ++, ewes had lower *BMP15* but not *GDF9*, mRNA levels. This finding is consistent with the overall hypothesis that the higher ovulation rate in BB ewes is linked to the timing of onset of LH-responsiveness in developing follicles and this in turn might be regulated by oocyte-derived BMP15.

In conclusion, using a qPCR approach, these studies confirm for sheep that the expression of *GDF9* and *BMP15* mRNA in COC is specific to oocytes and is tightly regulated and influenced by follicular health. Additionally, we confirm that a greater proportion of ovarian follicles ≥ 1 mm diameter in BB ewes attain LH-responsiveness earlier in development compared with that in ++ ewes. This study has established that there are no genotype effects of FSH-responsiveness in follicles. A major new finding is that oocytes from non-atretic follicles, FSH-responsive follicles and presumptive pre-ovulatory follicles (i.e. LH-responsive) in the BB ewes expressed less *BMP15* mRNA compared with that in wild types. Therefore, we propose that the common pathway to higher ovulation rates in BB ewes is similar, at least in part, to that in heterozygous *BMP15*-mutant ewes and that the oocyte plays a role in this pathway.

Materials and Methods

All experiments were performed with the approval of the Invermay Animal Ethics Committee in accordance with the 1999 Animal Welfare Act (Part 6) of New Zealand. All animals had access to pasture and water *ad libitum*.

Animals

The animals in this study were 5–7-year-old Booroola Romney wild-type (+++; $N=4$) or Booroola Romney homozygous *BMP1B*-mutant ewes (BB; $N=3$). The ++ and BB ewes were derived from mating animals of known pedigrees. The ovaries of all animals were collected 24 h after the onset of a follicular phase, as induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment on days 10–11 of the luteal phase of the oestrous cycle.

Ovarian collection and recovery of COC and GC

Ovaries were extracted, weighed and collected into saline (0.9%) containing 20 mM Hepes buffer (pH 7.4). Thereafter, all follicles ≥ 1 mm diameter and CL were dissected at room temperature in DMEM supplemented with 20 mM Hepes buffer, 0.2 mM 3-isobutyl-methyl-xanthine (Sigma Chemical Co.) and 0.1% (w/v) BSA ($>97\%$ pure; ImmunoChemical Products Ltd, Auckland, New Zealand). The numbers and weights of CL were recorded. Each individual follicle was transferred into a dry petri dish, and the diameter was measured using a graticule in the eyepiece of the dissecting microscope before puncture for release of intra-follicular contents. The COC was then recovered in 2–4 μ l of follicular fluid, snap frozen and stored at -80°C until total RNA extraction. The remaining follicular fluid was carefully removed from the dish and stored at -20°C in 100 μ l PBS. Thereafter, 1 ml of DMEM was added to the petri dish, and the GC were scraped from the inner wall of the follicle. The number of GC per follicle was determined by haemocytometer and the health status of each individual follicle was determined as described earlier (McNatty *et al.* 1986). In brief, non-atretic follicles were those defined as having a vascularised theca interna, the absence of debris in follicular fluid and a normal-looking COC and $\geq 25\%$ of the maximum predetermined number of GC for a given diameter. Atretic follicles were considered to be those that fail one or more of the above criteria. In those atretic follicles, COC were collected for further analyses but GC were discarded.

For individual non-atretic follicles, the GC were centrifuged at 300 *g* at room temperature for 5 min, and the resulting cell pellets were resuspended in the aforementioned DMEM medium to a final concentration of 60 000 cells per incubation. Cells were incubated with or without FSH (0, 10, 100 and 1000 ng/ml) or hCG (100 ng/ml) in triplicate in a final volume of 600 μ l in 48-well culture plates incubated in a water bath at 37°C for 45 min. When insufficient numbers of cells were collected to undertake all treatments (e.g. in follicles 1–2.5 mm diameter), the minimum treatment regime was the inclusion of a control, a high dose of FSH (1000 ng/ml) and hCG (100 ng/ml) in duplicate. Under this regime, over 70% of all non-atretic follicles 1–2.5 mm diameter and $>95\%$ of all non-atretic follicles >2.5 mm were analysed for cAMP production in response to treatment. After the 45 min incubation period, samples were heated to 80°C for 15 min and then stored at -20°C until assayed for cAMP.

FSH and LH reagents

The ovine FSH preparation was a highly purified in-house ovine reagent (oFSH Wal; McNatty *et al.* 2009). This reagent had a bioactivity of $1.4 \times \text{USDA-oFSH-19-SIAFP}$ or 33 000 IU/mg when the human FSH International Reference Preparation 78/549 was used as a standard in a radio-receptor assay (Cheng 1975). The level of LH contamination was $<0.002\%$ as determined by bioassay. The LH reagent was the hCG preparation CR121 (13 450 IU/mg) supplied by the National Institutes of Child Health and Development, Bethesda, MD, USA. The reason for using hCG is that it is devoid of FSH

activity, whereas most LH preparations have residual FSH contamination.

Cyclic AMP RIA

The cAMP assay was performed as described previously (McNatty *et al.* 2009), and had a detection limit of 0.2 pm/10⁶ cells and the intra- and inter-assay coefficients of variation were <9%. The in-house primary antibody had a cross-reactivity of 9% with dibutyl-*c*-AMP, <0.001% with *c*GMP and ≤0.0001% with AMP, ADP or ATP.

Analyses of GDF9 and BMP15 mRNA

All reagents used and procedures performed were according to the manufacturers' instructions. The expression levels of *GDF9*, *BMP15* and *RPL19* (housekeeping gene) mRNAs and of *GJA4*, *GJA1* and *RPL19* were determined in triplex reactions using a Taqman qPCR method. The expression levels of *FSHR* and *RPL19* were determined in singleplex reactions using a SYBR green qPCR method.

Total RNA was extracted from frozen individual COC using reagents from the ArrayPure Nano-Scale RNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA). To remove any genomic DNA, the kit protocol included the incubation of each sample with DNase I for 30 min. Following total RNA extraction, each sample was re-suspended in 10 µl of UltraPure DNase/RNase-free distilled water (Invitrogen). Owing to limited template, the entire DNase-treated total RNA sample was reverse-transcribed using reagents including oligo(dT)₂₀ from the SuperScript VILO cDNA Synthesis kit (Invitrogen).

Primers and Taqman probes for all genes were designed for multiplex (Taqman) and singleplex (SYBR green) qPCR using the computer package 'Beacon Designer' (Premier Biosoft International, Palo Alto, CA, USA) and are listed in Table 2. Taqman probes and primers were manufactured by Sigma–Proligo (supplied by Proligo-France SAS 1, Paris France and Proligo-Singapore Pte Ltd, Helios, Singapore) and Invitrogen respectively.

A control experiment was performed to determine the localisation of gene expression of *GDF9* and *BMP15* in different cell types of the COC (i.e. cumulus cells and denuded oocytes). Single COC were extracted from sheep ovaries obtained from a nearby abattoir. Cumulus cells were separated from oocytes by repeated pipetting. Single cumulus cell masses and denuded oocytes were separately washed three times in PBS, and total RNA was extracted and cDNA synthesised as described earlier. For quantification of the expression levels of *GDF9* and *BMP15* mRNA, or of *GJA4* and *GJA1* mRNA, triplex reaction mixes were prepared containing primers and Taqman probes for ovine *GDF9*, *BMP15* and *RPL19* genes or ovine *GJA4*, *GJA1* and *RPL19* genes, respectively, at optimised concentrations (Table 2) and reagents supplied in the Brilliant Multiplex QPCR Master Mix kit (Stratagene, La Jolla, CA, USA). For quantification of the expression levels of *FSHR* mRNA, singleplex reaction mixes were prepared containing primers for ovine *FSHR* or *RPL19* genes at optimised concentrations (Table 2) and reagents supplied in the 'Brilliant SYBR Green QPCR Master Mix' kit (Stratagene). Samples were prepared in duplicate by adding an aliquot of neat cDNA (3.12 µl) into the prepared reaction mix (total volume of 52 µl) and then transferring two 25 µl aliquots to adjacent 0.1 ml strip tubes (Corbett Research Ltd, Mortlake, NSW, Australia). The amplification reaction was run on a Rotor-Gene 6000 multiplexing system (Corbett Research Ltd) using the following conditions: 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Before analysis, serial dilutions (1:1 to 1:64) of two samples were made in appropriate singleplex or triplex reactions to validate PCR reaction efficiency for each gene. This included the calculation of the line of best fit (slope ± 0.1) for all genes tested when ΔΔCT was plotted against log (input total RNA), as well as comparing CT values (<0.5 cycles different) for identical samples for all mRNA transcripts in singleplex and multiplex reactions.

For quantification of the expression levels of *GDF9* and *BMP15* mRNA in experimental samples, a triplex reaction mix was prepared using 1.04 µl of neat COC-derived cDNA and amplified under the same conditions as described earlier. Controls were incorporated in every run and included

Table 2 Sequence information and final concentrations (nM) of primers and Taqman probes for sheep *GDF9*, *BMP15*, *GJA4*, *GJA1*, *FSHR* and *RPL19* genes for use in either triplex Taqman (^λ*GDF9*, *BMP15* and *RPL19*; ^ψ*GJA4*, *GJA1* and *RPL19*) or singleplex SYBR green (^δ*FSHR* and *RPL19*) quantitative PCR reactions.

Gene	Taqman probe (5'–3')	nM	Primers (5'–3')	nM
<i>GDF9</i>	(6FAM)AGTCTCAGCCTCAGATTCCAACGCAGTCCTA(BHQ1)	50 ^{λψ}	F-ATTAGCCTTGATTCTCTGCCTTCTAG R-GTGTCTCCACCTAAATGGTTCAG	200 ^λ 200 ^λ
<i>BMP15</i>	(HEX)AGAATGTCCCTCAGCCTTCCTGTGTCCCT(BHQ1)	50 ^{λψ}	F-AACCTGTCTCAGTGTCTGGTG R-AGATACTCCCATTTGCCCTCAATCAG	200 ^λ 200 ^λ
<i>GJA4</i>	(6FAM)TCCTTCTCGTCAGCACGCCACCC(BHQ1)	100 ^ψ	F-TTCCCATCTCCACATCCG R-TCCGCGCCGAGACAGGTAG	200 ^ψ 200 ^ψ
<i>GJA1</i>	(HEX)CGGCACTCAAGCTGAATCCATAGATGTACCACT(BHQ1)	50 ^ψ	F-TCTTCAAGTCTGTCTTCGAGGTG R-CTGATCGGGGACAGGATC	200 ^ψ 50 ^ψ
<i>FSHR</i>			F-AGGACAGCAAGGTGACAGAGATG R-GTAGTTTGGGACAGTTGGAGAAC	100 ^δ 100 ^δ
<i>RPL19</i>	(CY5) TTCTCATCCTCCTCATCCACGTTACCTTCTCGG(BHQ3)	50 ^{λψδ}	F-TAAGCGAAAGGTACTGCCAATG R-TTCTTAGATTCACGGTATCGTCTGAG	200 ^{λψδ} 200 ^{λψδ}

HEX, 6FAM, CY5 are fluorophores, and BHQ1 and 3 are quenchers from Sigma–Proligo.

randomly picked samples that underwent reverse transcription-PCR with the exclusion of Superscript III/RNaseout enzyme mix to examine the effectiveness of DNase treatment, and reactions that omitted addition of template.

Quantification of samples was calculated using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001). The expression levels of *GDF9*, *BMP15*, *GJA4*, *GJA1* and *FSHR* from samples from the control experiment (i.e. cumulus cells and denuded oocytes) were corrected for *RPL19* mRNA levels. The expression levels of *GDF9* and *BMP15* in the experimental samples (i.e. COC) were relative to a calibrator sample (cDNA from ovine ovary) and not corrected for *RPL19* mRNA levels as expression of this gene was not specific to oocytes. Therefore, *GDF9* and *BMP15* mRNA levels are per oocyte and not relative to total RNA input. The ratios of *GDF9*:*BMP15* mRNA in individual COC was calculated by $1:2^{-(C_{T_{GDF9}} - C_{T_{BMP15}})}$.

Statistical procedures

Levels of mRNA are expressed as means, and the variance range is estimated by evaluating the $2^{-\Delta\Delta C_T}$ value plus and minus the s.d. leading to asymmetric error distribution (Livak & Schmittgen 2001). The effects of follicular diameter and genotype on GC number or expression levels of *GDF9* and *BMP15* mRNA were assessed by ANOVA. As there were no effects of follicular diameter or interactions with respect to *GDF9* and *BMP15* mRNA, the comparisons of genotype levels between non-atretic follicles, atretic follicles and FSH- or LH-responsive follicles were by done by Student's *t*-test or Mann-Whitney *U* test if the variances were not equal. The comparisons between genotypes of the *GDF9*/*BMP15* mRNA ratios were also assessed by Mann-Whitney *U* test and the counts of ratios > 1.1 were analysed by χ^2 test. The adequacy of a linear fit to the regression of *GDF9* and *BMP15* mRNA levels in individual COC from both genotypes was confirmed by a residual plot. GC producing > 10 pmol/ 10^6 cells/45 min when incubated with either FSH or hCG were considered to be gonadotropin-responsive; this value was $> 2 \times$ the overall SD response above that produced by cells without gonadotropin treatment. For analyses of cAMP data, the main effects of follicular diameter, FSH dose and genotype were assessed by ANOVA. The proportions of FSH- and LH-responsive GC with respect to genotype were assessed by χ^2 analyses.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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