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Bone morphogenetic proteins and folliculogenesis: lessons from the Booroola mutation

C. J. H. Souza[1]*, B. K. Campbell[2], A. S. McNeilly[3] and D. T. Baird[1]

[1]*Department of Reproductive and Developmental Sciences, University of Edinburgh, Centre for Reproductive Biology, 49 Little France Crescent, Edinburgh EH16 4SB, UK;* [2]*School of Human Development, University of Nottingham, D Floor East Block, Queen's Medical Centre, Nottingham NG7 2UH, UK; and* [3]*MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, 49 Little France Crescent, Edinburgh EH16 4SB, UK*

The Booroola phenotype is associated with a point mutation in the kinase domain of the bone morphogenetic protein receptor 1B (BMPR1B), and is characterized by 'precocious' differentiation of ovarian follicles, leading to the production of large numbers of ovulatory follicles that are smaller in diameter than wild-type follicles. These smaller follicles attain differentiation markers, such as expression of mRNA for P450aromatase and inhibin-βA subunit, granulosa cell LH receptors and aromatase activity, earlier than follicles from wild-type ewes. However, the preovulatory follicles from mutant ewes collectively secrete similar quantities of oestradiol, androstenedione and inhibin A in exactly the same pattern as wild-type ewes, which result in similar concentrations of FSH. The available evidence strongly indicates that the Booroola mutation exerts its action at the ovary rather than by altering gonadotrophin secretion. The bone morphogenetic protein (BMP) receptors and putative ligands are ubiquitously expressed within the ovary and BMPs seem to be involved in the paracrine regulation of FSH action. Thus, if the mutation is causing a reduction in BMPR1B signalling, it may act on an inhibitor of follicle differentiation. Further research in this area will concentrate on the elucidation of the natural ligands for BMPR1B at different stages of follicle development and examine the effect of BMPR1B mutation on the downstream signalling cascade

Introduction

The Booroola phenotype (FecB) is characterized in ewes by increased ovulation rate and litter size, which are associated with the 'precocious' development of a large number of antral follicles that are smaller than wild-type follicles. This phenotype has a classical Mendelian pattern of inheritance and was presumed to be the result of a single gene or closely associated

*Present address: EMBRAPA Genetic Resource and Biotechnology, Parque Estação Biologica s/n, Final W5 Norte, CEP 70770-900, Brasilia, Brazil
Email: csouza@cenargen.embrapa.br

cluster of genes. The putative gene has additive effects on ovulation rate and is dominant for litter size and was named Booroola after the Australian farm where it was first identified (Davis *et al.*, 1982; Bindon, 1984). On the basis of segregation of the ovulation rate in Merino and Romney flocks, the genotypes in ewes have been classified as homozygous non-carrier (++) with an ovulation rate of two or fewer, heterozygous carriers (B+) with an ovulation rate of three or four and homozygous carriers (BB) with five or more ovulations per cycle (Davis *et al.*, 1982). The phenotype has been associated with a single point mutation in the kinase domain of the gene coding for the bone morphogenetic protein receptor 1B (BMPR1B) (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001).

The bone morphogenetic proteins (BMPs) are very important in key steps in mammalian development as can be inferred from rodent models that are deficient for ligands, receptors and intracellular signalling proteins (for a review, see Goumans and Mummery, 2000). These effects can be profound, as in the BMP-4 null mice which fail to develop beyond the early stages of gestation (Lawson *et al.*, 1999), or mild, as in mice lacking BMP-6 function which show minor delay in skeletogenesis in late gestation but are viable and fertile, and newborn and adult knockout mice are indistinguishable from wild type (Solloway *et al.*, 1998).

Mice deficient for the BMPR1B are viable and show skeletal defects that are largely restricted to digit formation (Baur *et al.*, 2000; Yi *et al.*, 2000). However, the BMPR1B is essential for multiple aspects of female fertility, and knockout mice have irregular oestrous cycles, impaired pseudopregnancy response, defects in cumulus cell expansion that prevent fertilization *in vivo*, decreased aromatase production in granulosa cells and failure in endometrial gland formation (Yi *et al.*, 2001).

Booroola phenotype during fetal life

The Booroola mutation is associated with several altered phenotypes during fetal life (for a review, see McNatty *et al.*, 1995). It causes delay in development, and fetuses carrying the mutation have lower body weight per gestation age (40, 70, 90 and 135 days of age). Female fetuses have lighter ovaries and adrenal glands at late gestation (day 95), and the difference in ovarian size persists up to day 135 (Smith *et al.*, 1993). The FecB mutation is also associated with retarded development of the heart (day 28), mesonephros (days 30–40) and ovary (from day 30 to early neonatal life). The ovary of BB animals has fewer oogonia (days 30–40), primordial follicles (days 75–90) and growing follicles (day 120 to 6 weeks after birth) (McNatty *et al.*, 1995). However, none of these differences in development, apart from those of the ovary, apparently persist to term (day 145).

Non-reproductive adult phenotype

The effect of the Booroola mutation on the development of other systems was investigated in Scottish Blackface × Merino ewes of 10 and 22 months of age. The internal organs of 17 ewes homozygous for the mutation and 18 wild-type ewes were examined and weighed. There were no macroscopic differences in the organs examined; the total body weight and the mass of the heart, liver, lungs, kidneys and spleen were not significantly different between the genotypes. In contrast, the adrenal glands from both sides weighed significantly less in BB than ++ ewes ($P < 0.05$). ANOVA was performed to include other factors that could affect adrenal mass. The combined adrenal mass was influenced by genotype (2.33 ± 0.08 and 1.95 ± 0.08 g for ++ and BB ewes, respectively, $P < 0.05$) and age (2.44 ± 0.07 and 1.84 ± 0.08 g for animals of 10 and 22 months of age, $P < 0.05$), but the interaction with genotype was not significant (Souza and Baird, 2001). The effect of the mutation on adrenal gland function was evaluated by measurement of basal cortisol secretion and secretion after an

ACTH challenge, before and after dexamethasone suppression, but no effect of the Booroola mutation was observed. These findings indicate that the Booroola mutation affects the size of the adrenal glands and indicate that the mutated gene could be important in the development of other organs in the body in addition to the main phenotype in the ovary. However, the function of the adrenal glands is not compromised or is compensated for in the mutant adults.

Phenotype in reproductive organs

The concentration of immunoreactive GnRH in hypothalamic and extra-hypothalamic areas of the brain from intact and ovariectomized (OVX) ewes are similar among the genotypes for the Booroola mutation (Gale *et al.*, 1988). The secretory pattern and GnRH concentration in the portal blood in OVX ewes (McNatty *et al.*, 1993) and GnRH-induced gonadotrophin secretion in OVX ewes in which the hypothalamic–pituitary stalk has been disconnected (HPD) are not influenced by genotype (McNatty *et al.*, 1991a). Moreover, when HPD ewes primed with equine chorionic gonadotrophin (eCG) are induced to ovulate by a pulsatile regimen of GnRH, the difference in the ovulation rate between BB and ++ genotype is maintained (McNatty *et al.*, 1993). All of these studies indicate that the Booroola mutation exerts its action 'downstream' from the hypothalamus.

The GnRH receptor in pituitary gland homogenates from intact and OVX ewes has a single class binding-site, the binding characteristics of which are not influenced by the Booroola genotype (Fleming *et al.*, 1990). In pituitary glands obtained from ++ and BB intact ewes during the mid-luteal phase, OVX ewes and ovary-intact or OVX-HPD ewes given the same regimen of pulsatile GnRH, no Booroola-specific differences were detected in the number or size of mRNA transcripts for alpha gonadotrophin, FSHβ and LHβ genes. In addition, the genotypes have similar relative amounts of mRNA encoding these genes and pituitary content of FSH or LH (Fleming *et al.*, 1995). Studies using immunohistochemistry also show no differences in the total number of pituitary cells, pituitary volume, numbers or diameters of FSHβ or LHβ immunostaining cells (Heath *et al.*, 1996).

There is no difference in the concentration of LH in blood or in the anterior pituitary gland between ewes carrying the Booroola mutation and control ewes (Robertson *et al.*, 1984; McNatty *et al.*, 1987). The data for FSH are controversial as some studies report differences among genotypes in FSH concentration during various stages of the oestrous cycle and anoestrus (Bindon, 1984; McNatty *et al.*, 1987, 1989a), whereas other reports show no difference (McNatty *et al.*, 1991b; Boulton *et al.*, 1995; Souza *et al.*, 1997). Nevertheless, there are several lines of evidence indicating that despite the trivial difference in FSH concentrations sometimes observed, the characteristic phenotype of the Booroola mutation can still be observed under similar gonadotrophin milieu. Ovulation induction during anoestrus with a combination of progestagen and eCG resulted in ewes retaining the difference in ovulation rate between genotypes (Souza *et al.*, 1994). Chronically hypophysectomized ewes induced to ovulate by a standard combination of eCG and hCG injections maintain the genotype difference in ovulation rate (Fry *et al.*, 1988). More recently, a study using Booroola ewes treated with a potent GnRH antagonist for 3 weeks to render the animals hypogonadotrophic and infused with the same gonadotrophin regimen designed to mimic the normal pattern of FSH and LH during the follicular phase retained the difference in ovulation rate and also the characteristic phenotype of smaller ovulatory follicles and corpora lutea (Campbell *et al.*, 1996).

Together, these observations provide strong evidence that the Booroola mutation does not act through pituitary function. However, it is possible that the mutation could have an effect on the pituitary gland as the BMPR1B is shown by PCR to be expressed in the sheep pituitary

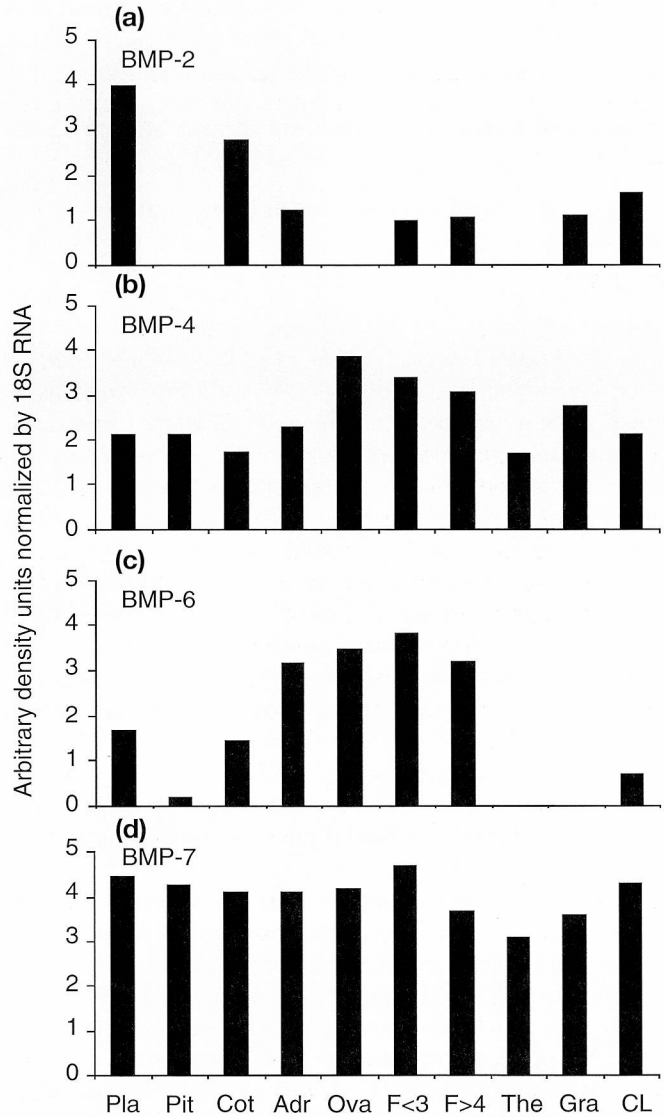

Fig. 1. Expression of bone morphogenetic protein (BMP) mRNA ((a) BMP-2 mRNA; (b) BMP-4 mRNA; (c) BMP-6 mRNA and (d) BMP-7 mRNA) in sheep tissues by northern blot analysis (15 μg total RNA loaded per lane). Adr: adrenal; Cot: cotyledon; CL: corpus luteum; F < 3: follicle < 3 mm; F > 4: follicle > 4 mm; Gra: granulosa > 3 mm; Ova: ovary; Pit: pituitary; Pla: placenta; The: theca > 3 mm.

gland (Wilson *et al.*, 2001). BMP-7, a known ligand for the mutant receptor, increases the expression of FSHβ RNA in rodent *in vitro* systems (Huang *et al.*, 2001) and BMP-4 and -7 are expressed in the sheep pituitary gland (Fig. 1). Nevertheless, differences in gonadotrophin secretion cannot account for the ovarian phenotype of the Booroola mutation.

Table 1. Preovulatory follicles and corpora lutea in Booroola mutant (B+) and wild-type (++) ewes

Characteristic	B+	++	*P* value
Oestrogenic follicles per ewe	2.6 ± 0.3	1.1 ± 0.1	< 0.001
Granulosa cells per follicle (10^6)	0.68 ± 0.06	1.36 ± 0.19	< 0.001
Total granulosa cells per ewe (10^6)	1.69 ± 0.20	1.52 ± 0.27	NS
Corpora lutea per ewe	2.5 ± 0.3	1.1 ± 0.1	< 0.01
Luteal tissue per corpus luteum (g)	0.12 ± 0.03	0.29 ± 0.07	< 0.001
Total corpus luteum mass per ewe (g)	0.30 ± 0.04	0.31 ± 0.01	NS

Adapted from Baird *et al.* (1982).

Ovarian phenotype

The most consistent phenotypes resulting from the Booroola mutation are the size and number of the ovulatory follicles, which were reported first in Merino ewes (Baird *et al.*, 1982) and subsequently confirmed by several groups in flocks in which the Booroola mutation was introduced into different breeds (Driancourt *et al.*, 1986; McNatty *et al.*, 1986a; Souza, *et al.*, 1994). The more numerous oestrogenic follicles of the Booroola ewes are smaller and have fewer granulosa cells than those of ++ ewes (Table 1). Nevertheless, the total number of granulosa cells from the combined ovulatory follicles is similar in mutant and wild-type sheep. The smaller preovulatory follicles also form lighter corpora lutea, but the total mass of luteal tissue in the ovaries is similar between the genotypes (Baird *et al.*, 1982). These initial observations were confirmed and expanded in a series of very elegant experiments performed by the Wallaceville group (for a review, see Montgomery *et al.*, 1992).

The total number of granulosa cells from oestrogenic follicles per animal is similar among the genotypes, as are the secretions of ovarian steroid and immunoreactive inhibin obtained from single samples from anaesthetized animals (McNatty and Henderson, 1987; McNatty *et al.*, 1992). In Merino ewes with ovarian autotransplants, there is no difference between genotypes in the rate of secretion of oestradiol and progesterone on day 10 of the luteal phase and at various times during the follicular phase (Tsonis *et al.*, 1988). Similar features are also observed regarding the population of corpora lutea among the genotypes. Although BB ewes have individual corpora lutea that are lighter and contain fewer cells per corpus luteum, the total luteal mass, total number of cells in the corpus luteum and concentration of progesterone in the plasma are similar to those of the ++ ewes (Niswender *et al.*, 1990). We examined this question in more detail using ewes with an ovarian autotransplant, which allowed long-term ovarian blood sampling and the use of ultrasonography to track the growth of individual follicles. These studies showed that the dynamics of follicle development in ewes with and without the Booroola mutation are similar during the follicular and early luteal phases of the oestrous cycle, but that the follicles ovulate and achieve dominance at a smaller diameter. The transplant model has enabled a very clear demonstration that, despite having more follicles, mutant ewes secrete similar quantities of oestradiol, androstenedione and inhibin A, in exactly the same pattern as wild-type ewes. The similarity in ovarian secretion of oestradiol and inhibin A explains the observation that FSH concentrations in the two groups were very similar, which is in contrast to the often repeated assertion that FSH concentrations are higher in mutants and strongly indicates that the mutation acts at the ovary to modulate gonadotrophic signals (Souza *et al.*, 1997). Thus, despite significant differences in ovulation rate and ovarian morphology, the genotypes appear to secrete similar amounts of the key components of the hormonal feedback system that regulates gonadotrophin secretion by the hypothalamus–pituitary axis.

The follicles in the Booroola carrier ewes mature at a smaller diameter than do follicles in non-carrier ewes, and influence the basal content of cAMP and gonadotrophin-stimulated cAMP response *in vitro* in follicles from the early antral stage onwards (McNatty *et al.*, 1986b). However, the difference in second messenger activity does not seem to be related to either FSH or LH receptor binding characteristics on granulosa or theca and luteal cells, respectively (McNatty *et al.*, 1986c, 1989b). LH receptors were found in granulosa cells from follicles of smaller diameter in carrier ewes than in non-carrier ewes (3.0–4.5 mm for BB/B+ and $\geq$ 5 mm for ++) (McNatty *et al.*, 1986c).

Similarly, examination of expression of mRNA cytochrome P450 aromatase, 17α-hydroxylase, inhibin α (Inhα), β_A (Inhβ_A) and β_B (Inhβ_B) subunits and LH receptor in Scottish Blackface Merinos carrying the Booroola mutation showed that the main genotypic differences were an increase ($P < 0.001$) in aromatase and Inhβ_A expression in medium sized follicles of 2–4 mm in diameter (Campbell *et al.*, 1998). However, there was also a decrease ($P < 0.05$) in Inhβ_B expression in small follicles and an increase ($P < 0.001$) in 17α-hydroxylase expression in small follicles of Booroola sheep. In non-oestrogenic follicles in BB ewes, Inhβ_B expression in granulosa was lower and expression of thecal LH receptor and 17α-hydroxylase was higher ($P < 0.05$). Expression of LH receptor in the granulosa cells was not examined in this study. Overall, these results indicate that, in addition to changes in the patterns of expression that can be attributed to precocious differentiation of the granulosa cells (aromatase and Inhβ_A), the FecB gene results in changes in the pattern of expression of other differentiation markers in both granulosa and theca cells (Inhβ_B, LH receptor, 17α-hydroxylase) which may be involved in a common paracrine pathway to regulate follicular development.

Culture of granulosa cells *in vitro* from follicles at similar stages of maturation revealed no significant differences in cAMP synthesis after stimulation with FSH, LH, forskolin, cholera or pertussis toxin in the different genotypes and similar catabolism rate of cAMP (Henderson *et al.*, 1987; McNatty *et al.*, 1989b, 1990), indicating that the Booroola mutation has no direct influence on gonadotrophin binding and second messenger generating systems.

In follicles of $>$ 1 mm in diameter, granulosa cells are the only source of oestradiol and the capacity of these cells to aromatize androgens to oestradiol was at its highest in follicles of different diameter according to the FecB genotype (3.0–3.5 mm, 3.0–5.5 mm and 5 mm in diameter for BB, B+ and ++ ewes, respectively) (McNatty and Henderson, 1987). However, the steroid production of the theca cells, mainly androstenedione and to a lesser extent progesterone and testosterone, was not influenced by the Booroola genotype (McNatty and Henderson, 1987). Short-term culture (4 h) of small intact follicles, between 30 and 60% of the putative preovulatory size (2–4 mm in ++ and 1.5–3.0 mm in B+) with or without FSH showed that the granulosa cells of mutant ewes proliferate more in the presence or absence of gonadotrophin, whereas no differences were found in the theca cell layer. When large intact follicles with a diameter of $\geq$ 80% of the preovulatory size were cultured in a perfusion system for 24 h, no difference between genotypes was found in the oestradiol production after FSH stimulation (Driancourt, 1991). Granulosa cells from both genotypes cultured *in vitro* using a system that prevents spontaneous luteinization show that cells from mutant ewes have higher aromatase activity and are more responsive to FSH than cells from control ewes (Webb *et al.*, 1995).

The Booroola ovarian phenotype is not only restricted to the size and number of ovulatory follicles. Histological examination demonstrated that oocytes were larger in BB versus ++ preantral follicles. Oocytes from B+ follicles gain the ability to resume meiosis at a smaller size and a higher proportion of them reach metaphase II, irrespective of their size when compared with wild-type follicles. In addition, the developmental rate of eggs after IVF was also affected by genotype: B+ oocytes originating from follicles of 1.0–3.5 mm in diameter

had a greater ability to develop to the blastocyst stage than did ++ oocytes. These findings indicate that the Booroola mutation, in addition to its effects on granulosa cell maturation, affects oocyte development and function (Cognie *et al.*, 1998).

The effect on the paracrine regulation between the oocyte and the granulosa cells could be mediated by members of the transforming growth factor β (TGFβ) superfamily, such as growth differentiation factor 9 (GDF-9) or BMP-15 (for a review, see Eppig, 2001). In sheep, GDF-9 is exclusively expressed by the oocyte (Bodensteiner *et al.*, 1999) and seems to use the BMP signalling pathway. GDF-9 in rats binds to BMPR2 and can use both BMPR1A and BMPR1B for signal transduction, although BMPR1B seems to be the preferred type one receptor, and is threefold more active than BMPR1A (Vitt *et al.*, 2002). All BMP receptors are expressed in sheep, in follicles from the primary to large antral stage (Souza *et al.*, 2002). Another possible candidate for this action is BMP-15 which when biologically inactive causes the Inverdale phenotype and is also expressed in the ovine oocyte (Galloway *et al.*, 2000). BMP-15 (GDF-9b) is closely related to GDF-9 (Vitt *et al.*, 2002) and might use a similar signalling pathway as indicated by the additive effect on ovulation rate in Booroola Inverdale crosses (Davis *et al.*, 1999).

BMP system in the sheep ovary

The presence of BMPs and their receptors has been reported in the ovaries of a number of species including mice and rats (Shimasaki *et al.*, 1999, 2002; Elvin *et al.*, 2000). We have recently investigated the location of BMP receptors in sheep ovaries by immunohistochemistry. Strong immunostaining for BMPR1A, BMPR1B and BMPR2 was present in the granulosa cell layer of follicles from the primary to late antral stages of development. Staining was also observed in the oocyte, corpus luteum, ovarian surface epithelium and, to a lesser extent, the theca layer of antral follicles (Souza *et al.*, 2002). The sheep ovary also expresses the activin receptors type 2 and type 1B (ALK4) in a pattern remarkably similar to that observed for the BMP receptors (C. J. H. Souza, A. S. McNeilly and D. T. Baird, unpublished).

The potential ligands for the BMP receptors are also expressed in the sheep ovary (Fig. 1); BMP-4 and -7 are highly expressed in most of the tissues investigated. BMP-2 and -6 are expressed at a lesser intensity but nevertheless both are present in the sheep ovary, and BMP-6 is probably expressed in the oocyte as we observed expression in RNA from intact follicles but not in granulosa or theca cell RNA. All of these receptors can potentially interact with one or more of the BMPs expressed in the sheep ovary and studies on the cellular expression of BMP RNA and protein would help to clarify those interactions. Thus, the sheep ovary contains the signalling system that affects the ovarian cell in rodents.

Studies using *in vitro* culture of rat granulosa cells (for a review see, Shimasaki *et al.*, 2002) show that all BMPs tested inhibit progesterone secretion, whereas the secretion of oestradiol was stimulated by BMP-4 or -7, but unaffected by BMP-6 or -15. In sheep, BMP-2 enhanced oestradiol and inhibin A production without affecting the proliferation of the granulosa cells from immature follicles of 1–3 mm in diameter cultured in serum-free medium containing FSH. This finding indicates that BMPs may have a role in the differentiation of granulosa cells by enhancing the action of FSH (Souza *et al.*, 2002). The addition of GDF-5 and BMP-4 in a dose-dependent manner inhibits progesterone secretion by granulosa cells recovered from immature follicles cultured *in vitro*, regardless of the presence of FSH. In this culture system, Booroola granulosa cells are less responsive than wild-type cells to GDF-5 and BMP-4 (Mulsant *et al.*, 2001), whereas activin A or TGFβ have the same efficiency in reducing progesterone secretion by ovine granulosa cells *in vitro* from both genotypes (Monget *et al.*, 2002), indicating that the mutation is responsible for a partial decrease in function

of BMPR1B. Another possibility is an effect on the bioavailability and the diffusion of the BMPs in the ovary as both the membrane bound antagonist BAMBI and Chordin are showed by PCR to be expressed in the sheep ovary (C. J. H. Souza, A. S. McNeilly and D. T. Baird, unpublished).

Conclusions

The Booroola mutation causes 'precocious' differentiation of ovarian follicles, which leads to the production of more but smaller ovulatory follicles than in the wild type. This is reflected in the smaller diameter that follicles attain differentiative markers, such as expression of mRNA for P450aromatase and inhibin-βA subunit, granulosa cell LH receptors and aromatase activity.

The available evidence strongly indicates that the FecB mutation exerts its action at the ovary rather than through altered amounts of gonadotrophin stimulation. This is reflected by the fact that endocrine and follicular wave growth patterns are not altered in mutants and that differences between genotypes persist after physiological gonadotrophin replacement in GnRH-antagonist suppressed animals. Furthermore, granulosa cells from animals carrying the FecB mutation are more sensitive to gonadotrophin stimulation *in vitro*.

Finally, the BMP receptors and putative ligands are ubiquitously expressed within the ovary and BMPs seem to be involved in the paracrine regulation of FSH action. Thus, if the mutation is causing a reduction in BMPR1B signalling, it may act on an inhibitor of follicle differentiation. Further research in this area is required to concentrate on the elucidation of the natural ligands for the BMPR1B at different stages of follicle development and examination of the effect of BMPR1B mutation on the downstream signalling cascade.

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