

Mechanisms of action of the principal prolific genes and their application to sheep production

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Abstract. The prolificacy variation in sheep makes it an excellent animal model to understand the mechanisms regulating ovulation rate. Identification of mutations responsible for the increased prolificacy of the Inverdale, Booroola, Javanese, Cambridge and Belclare sheep open new avenues of investigation for the paracrine control of folliculogenesis. To date, all known mutations are in genes from ligands or receptors of the transforming growth factor p superfamily, and point to the bone morphogenetic protein family of peptides as local regulators of ovarian follicle growth. The mechanism of action of the mutated genes is not fully understood, but results in the ovulation of a higher number of follicles with smaller diameter and fewer granulosa cells than that of the wildtype, thus speeding the differentiation of ovulatory follicles. Comparisons of the performance of Booroola-crossed flocks in different countries showed that carriers of the prolificacy mutation have higher ewe productivity but also higher perinatal mortality and lighter weight lambs. Their economic impact on the sheep industry depends on farm environment and management. Nevertheless, the diagnostic tests now available to identify the genetic mutations resulting in increased ovulation rate will simplify the introduction of these mutations and their monitoring in flocks for research and commercial purposes.

Extra keywords: Booroola, Inverdale, mutation, ovulation rate, prolific sheep.

Introduction

The ewe, with its wide range in litter size among different breeds and within breeds, is an ideal model for the study of follicular selection and its regulation. Prolificacy in sheep is determined mainly by the number of ova shed in each oestrous cycle, that is, the ovulation rate. The ovulation rate can be genetically regulated by several genes, as in the Romanov and Finnish-Landrace breeds, or alternatively by the action of a major gene or a small group of closely linked genes (Baird and Campbell 1998). Several putative major genes influencing the ovulation rate have been described in sheep over the last two decades. Following the identification of the Booroola gene in the Merino (Davis *et al.* 1982; Piper and Bindon 1982), the Thoka (Jonmundsson and Adalsteinsson 1985), Javanese (Bradford *et al.* 1986), Olkuska (Martyniuk and Radomska 1991), Belclare (Hanrahan 1991), Cambridge (Hanrahan 1991),

Inverdale (Davis *et al.* 1992), Woodlands (Davis *et al.* 2001) and Lacaune (Bodin *et al.* 2002) genes have now been identified.

The mapping of major genes affecting the ovulation rate in sheep and segregation studies (for review see Montgomery *et al.* 2001) pointed to a location on the X chromosome for the Inverdale and Woodlands genes, with the latter being maternally imprinted. The remaining loci have been identified as autosomic, with the Booroola gene located on chromosome 6 (Montgomery *et al.* 1994) and the Lacaune on chromosome 11 (Mulsant *et al.* 2003). The mapping of the Belclare gene was confounded by the possibility of involvement of more than one locus (Montgomery *et al.* 2001), while the other major gene locations remain unknown. Hence, a conservative estimate would predict at least six independent mutations affecting ovulation rate in sheep.

Identification of the mutated genes affecting ovulation rate in sheep

The first prolific sheep to have its genetic basis identified was the Inverdale, which results from mutations in the bone morphogenetic protein 15 (BMP 15) gene, also known as growth and differentiation factor 9b (GDF9b; Galloway *et al.* 2000). Two independent point mutations were identified causing the phenotype: a C to T transition at nucleotide position 871, introducing a premature stop codon in the place of glutamine at amino acid residue 291 of the unprocessed protein, called Hanna (*FecXH*), and a T to A transition at nucleotide position 896, substituting valine with aspartic acid at residue 299 of the unprocessed protein, called Inverdale (*FecXI*).

The mutation responsible for the Booroola phenotype has been identified by three independent laboratories as being a single point mutation in the kinase domain of the gene coding for the BMP receptor IB (Alk6). This is due to an A to G transition at position 746, substituting the glutamine present in the wildtype with an arginine at position 249 of the protein (Mulsant *et al.* 2001; Souza *et al.* 2001; Wilson *et al.* 2001). After the description of the Q249R mutation in the Booroola animals, several prolific breeds have been screened for this mutation and it has been identified as being responsible for the prolificacy of the Javanese, Garole (Davis *et al.* 2002) and Little-Tailed Han Sheep (Liu *et al.* 2003).

Recently, mutations in the GDF9 and BMP 15 genes have been identified in Cambridge and Belclare sheep (Hanrahan *et al.* 2004). The *FecG^H* (GH) mutation is a C to T transition at position 1184 of the GDF9 gene coding sequence replacing a serine for a phenylalanine at residue 77 of the mature peptide. The additional mutations are in the BMP 15 gene: the *FecXP* (XG, Galway) mutation is a C to T substitution at nucleotide 718, introducing a premature stop codon in the place of the glutamine at amino acid 239 of the unprocessed protein; the other mutation is *FecX^B* (XB, Belclare), a G to T transition at nucleotide 1100, substituting the serine residue at amino acid 99 of the mature protein (residue 367 of the unprocessed protein) with isoleucine. The Belclare sheep presents all 3 mutations but the XB is the most prevalent occurring in 71% of the animals, while the Cambridge flock does not have the XB mutation but presents the XG and GH mutations at high prevalence (Hanrahan *et al.* 2004).

Bone morphogenetic protein action in folliculogenesis

The identification of these mutated genes in prolific sheep point to the transforming growth factor β (TGF β) superfamily of peptides as paracrine regulators of ovarian follicular growth, especially the BMPs. The actions of BMP proteins in folliculogenesis have been reviewed in rodent models (Shimasaki *et al.* 2003). The BMP2 mRNA expression is localised in rat granulosa cells of follicles ranging from primary to antral stages, with enhanced expression as the follicles develop, reaching the highest signal in atretic follicles

(Erickson and Shimasaki 2003). Both BMP4 and BMP7 expression have been localised in the rat theca interstitial cells by *in situ* hybridisation in rat ovaries (Shimasaki *et al.* 1999). In the rat and mouse, BMP 15 and GDF9 are expressed by oocytes from primary follicles onwards, while BMP6 is expressed at high levels in immature and mature oocytes and at lower levels in the granulosa cells (Elvin *et al.* 2000; Erickson and Shimasaki 2003).

In sheep, the mRNA message of GDF9 is also located in oocytes from primordial to large antral follicles (Bodensteiner *et al.* 1999), whereas BMP 15 gene expression begins in oocytes from primary follicles (Galloway *et al.* 2000). The cellular localisation of GDF9 and BMP 15 proteins in the ovaries of lambs, identified using immunohistochemistry, established that the oocyte is the only intraovarian source of these growth factors in sheep (Juengel *et al.* 2002). Northern blot analysis of sheep tissues showed that BMPs 4 and 7 are highly expressed in granulosa and theca cells, BMP2 is expressed in the granulosa cells at a lesser intensity, and BMP6 is expressed in the oocyte (Souza *et al.* 2003).

The BMPs act through serine-threonine receptors with a single transmembrane domain, which exist as two subtypes, type 1 and type 2, with the latter being constitutively phosphorylated. Both types of receptors independently have a low affinity for the ligand, but together bind with a high affinity. Binding of a ligand to type 2 in concert with type 1 receptors leads to the formation of a heterotetrameric receptor complex and phosphorylation of the type 1 receptor. Once phosphorylated, the type 1 receptor interacts with downstream signaling proteins (Smad proteins), transphosphorylating one of the receptor-regulated Smad proteins (Smad 1, 5 or 8), which then heterodimerises with a common Smad (Smad 4) and moves to the nucleus where the Smad complex associates with nuclear transcription factors and activates transcription of target genes (Massague 1998; Miyazono *et al.* 2001).

The BMP receptors (BMPR) in sheep ovaries have been investigated using immunohistochemistry, which has shown strong expression of BMPR1 A, BMPR1B and BMPR2 in the granulosa cell layer of follicles from primary to late antral stages of development. Immunostaining was also observed in the oocyte, corpus luteum, ovarian surface epithelium and, to a lesser extent, in the theca layer of antral follicles (Souza *et al.* 2002).

The mechanism by which BMPs affect ovarian steroidogenesis is complex and not fully understood. The results of many studies show that the effects are different in a range of species and involve a range of ligands. *In vitro* studies of rat granulosa cells showed that BMPs 4, 6, 7 and 15, in the presence of FSH, reduce progesterone secretion, whereas oestradiol production is stimulated by BMPs 4 and 7, but remains unaffected by BMPs 6 and 15 (Shimasaki *et al.* 2003). This divergence in steroidogenesis may result from BMP7 enhancing aromatase activity, but suppressing steroidogenic acute regulatory protein (StAR) mRNA expression induced by FSH

(Lee *et al.* 2001). The effects of BMP6 on steroidogenesis are mediated by inhibiting StAR and side-chain cleavage expression, without affecting the expression of aromatase mRNA (Otsuka *et al.* 2001a). BMP6 also affected other FSH-responsive genes by inhibiting the expression of the inhibin/activin subunits (α , β A and β B) and the LH receptor by suppressing adenylate cyclase activity (Otsuka *et al.* 2001a). The effect of BMP 15 on granulosa steroid production, although similar to BMP6, is achieved by an inhibition of FSH-responsive genes owing to a reduction of FSH receptor mRNA expression (Otsuka *et al.* 2001b).

In sheep, granulosa cell culture of immature follicles (1-3 mm) with BMP2 in the presence of FSH, enhanced oestradiol and inhibin A production, without affecting cell proliferation (Souza *et al.* 2002), whereas BMP4 reduced progesterone secretion owing to a reduction in side-chain cleavage expression (Mulsant *et al.* 2001; Fabre *et al.* 2003).

Further studies involving sheep using a granulosa cell culture system that prevented spontaneous luteinisation (Campbell *et al.* 1996) have confirmed that the BMPs 2, 4 and 6 have no effect on granulosa cell proliferation, even in culture conditions where insulin-like growth factor (IGF) and FSH concentrations are low. No difference between BMPs 2, 4, and 6 were observed in terms of their ability to enhance gonadotrophin-induced oestradiol production, although a significant interaction between IGF-1 and the BMPs was observed (Fig. 1). Examination of the effect of

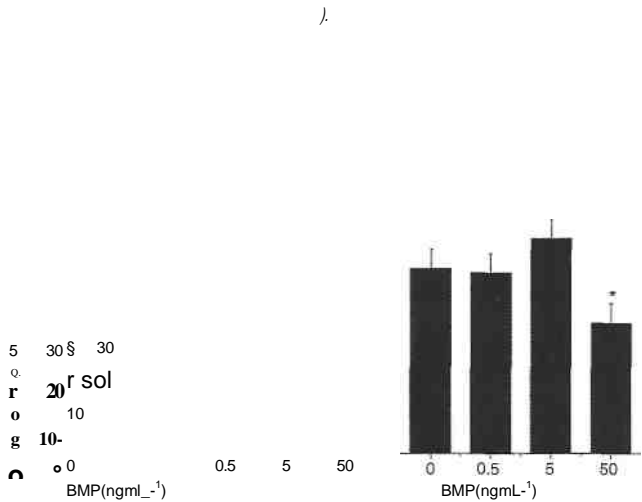


Fig. 1. Oestradiol production from sheep granulosa cells taken from immature follicles (1-3 mm) that were cultured in the presence 1 ngmL⁻¹ of FSH and varying concentrations of bone morphogenetic protein (BMP) and insulin-like growth factor (IGF-1). Panels (a-d) show data from follicles cultured with 0, 0.1, 1 and 10 ngmL⁻¹ IGF-1 respectively. Asterisk indicates significant difference ($P < 0.05$) compared with control.

BMPs on FSH-responsiveness showed that the BMPs did not increase the sensitivity of granulosa cells to FSH, acting to simply increase the magnitude of the response to a given dose. In marked contrast to granulosa cells, BMPs 2, 4 and 6 had an inhibitory effect on LH-stimulated androstene-dione production by cultured thecal cells, with BMP6 being less potent in this regard (Fig. 2). Furthermore, at very low doses, all BMPs stimulated proliferation of theca cells, even in the presence of IGF-1. Immunohistochemistry confirmed the expression of BMP6 protein in the oocyte, granulosa and theca cell layer of antral sheep follicles. Together, these results confirm that the action of the BMPs in monovulatory ruminants differs from those observed in polyovular rodents, and that the BMP system represents a major local regulatory system acting in concert with those already described (e.g. the IGF system) to modulate the proliferative and differ-entative action of gonadotrophins on ovarian somatic cells (B. K. Campbell, C. J. H. Souza, A. Skinner and D. T. Baird, unpublished data).

The BMPs also exhibit mitotic properties on rat granulosa cells *in vitro*. BMP7 increases thymidine incorporation and cell number (Lee *et al.* 2001). The effect of BMP 15 on granulosa mitotic activity is FSH-independent, suggesting that it is directly involved in controlling granulosa proliferation in preantral follicles during the FSH-independent stages of early folliculogenesis (Otsuka *et al.* 2001). A similar effect of BMPs was observed in sheep granulosa cell proliferation, with BMP4 (Mulsant *et al.* 2001; Fabre *et al.* 2003) and BMP 15 (McNatty *et al.* 2003) enhancing thymidine incorporation.

Growth and differentiation factor 9 in rat granulosa cultures stimulated proliferation of cells from both early antral and preovulatory follicles (Vitt *et al.* 2000). In rat granulosa cells cultured *in vitro* in the presence of FSH, GDF9 suppressed progesterone and oestradiol secretion (Vitt *et al.*

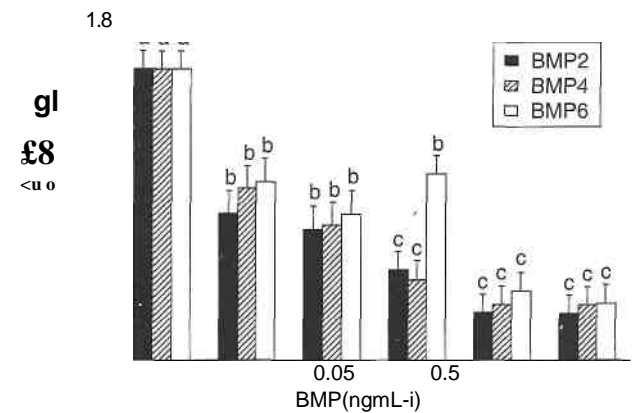


Fig. 2. Androstenedione production from sheep theca taken from immature follicles (1-3 mm) cultured with varying concentrations of bone morphogenetic protein (BMP) 2, 4 or 6. Different letters denote significant difference ($P < 0.05$).

2000) but increased inhibin A and B production (Roh *et al.* 2003).

Mechanism of action of the mutations increasing ovulation rate

A common characteristic of preovulatory follicles in prolific sheep is that their diameter at ovulation is smaller and they have fewer granulosa cells, developing into lighter corpora lutea than in the wildtype sheep. This is well documented in the Booroola Merino (Souza *et al.* 2003) and reported in heterozygous Inverdale ewes (Shackell *et al.* 1993). The mechanisms by which the mutated genes influence ovulation rate in sheep are not well understood. However, known ovarian phenotypes of the carriers allied to data from rodent models can offer some information about how these mutations could be acting in sheep folliculogenesis.

The main feature of prolific Booroola ewes is the precocious differentiation of ovarian follicles, which ovulate at a smaller diameter and in greater numbers than in the wild-type animals (Baird and Campbell 1998; Souza *et al.* 2003). In transfected HEK-293 cells, co-expression of BMPR1B and BMPR2 resulted in the induction of the activity of a BMP-specific luciferase reporter construct by BMP4. However, the response to BMP4 was abolished when cells were transfected with the BMPR2 and BMPR1B receptor with the Q249R mutation (Fabre *et al.* 2003). This reduction in receptor function is also illustrated by the fact that granulosa cells taken from small antral follicles of homozygous mutant ewes are threefold less responsive to the inhibition of progesterone production induced by BMP4 or GDF5, and less responsive to proliferation induced by BMP4 when compared with cells from wildtype ewes (Fabre *et al.* 2003).

Another possible mechanism for the Q249R mutation is the ablation of BMP6 biological function (Shimasaki *et al.* 2003). This notion is supported by some similarities between Booroola phenotypes and BMP6 action in rodent models, such as increased FSH activity without changes in FSH binding capacity, which might be mediated by a reduction in the inhibition of adenylate cyclase activity caused by BMP6 without changing FSH receptor expression. However, to date, no experimental evidence from sheep is available to corroborate this hypothesis.

The mutations in oocyte-expressed growth factors, BMP 15 and GDF9, are involved in increased prolificacy in heterozygous ewes and infertility in homozygous animals (Galloway *et al.* 2000; Hanrahan *et al.* 2004). In sheep, both growth factors are essential for ovarian follicular development (Juengel *et al.* 2002). Active immunisation against either BMP 15 or GDF9 caused arrest of follicular growth beyond the primary stage. Passive immunisation with anti-serum against BMP 15, before induced luteal regression, disrupted ovarian function resulting in ovulation failure,

while immunisation against GDF9 resulted in impaired luteal function (Juengel *et al.* 2002).

The proposed mechanism of action of the Inverdale mutation indicates that the substitution of a valine with aspartic acid in the mature peptide would prevent dimerisation (Galloway *et al.* 2000). Studies of transfected cells, which express recombinant BMP15, GDF9, or both, demonstrate that both can form homodimers when expressed individually, while heterodimers are produced when both are co-expressed. When GDF9 and BMP 15 are co-expressed, the processing of both proproteins are impaired as compared with that of the singly expressed proproteins, suggesting that the proprotein heterodimer is less susceptible to proteolytic cleavage than the individual homodimers. Stable transformants expressing BMP 15 with the Inverdale mutation alone, show that non-covalent dimers are formed even though the processing efficiency of mutant proprotein is significantly lower than that of the wildtype BMP15. In addition, when GDF9 and Inverdale BMP 15 are co-expressed, the processing and secretion of the mutant BMP 15 is abolished, and the processing of GDF9 is severely reduced (Liao *et al.* 2003), showing that the interaction between the Inverdale mutant BMP 15 and GDF9 occurs during post-translational processing because the mRNA expression of GDF9 is not affected (Liao *et al.* 2003). This is corroborated, at least at the transcriptional level, by the fact that ovaries of Inverdale homozygous ewes show GDF9 mRNA at similar levels to wildtype animals (Boden-steiner *et al.* 2000). However, to definitively confirm these findings in sheep, the expression pattern of GDF9 protein has to be investigated in the ovaries of Inverdale ewes. Further evidence of the interaction between oocyte-derived BMP 15 and GDF9 arises from the fact that animals with mutations in one allele of both genes have an even higher ovulation rate when compared with animals that are heterozygous to either mutation (Hanrahan *et al.* 2004).

The Hanna and Galway mutations in BMP 15 introduce a premature stop codon and probably result in no mature protein being processed (Galloway *et al.* 2000; Hanrahan *et al.* 2004). The Belclare mutation at serine 99 of the mature BMP 15 occurs in a residue that is highly conserved among members of the TGF β 3 superfamily, which is essential for receptor binding to the type 2 receptor (Hanrahan *et al.* 2004).

The GH mutation in the GDF9 gene occurs at a region of the molecule that is likely to be involved in binding to the type 1 receptor but it could also be affecting the biological activity by disrupting dimer stability (Hanrahan *et al.* 2004). Recent studies in transfected cells demonstrate that GDF9 binds first to BMPR2 (Vitt *et al.* 2002) and signalling is mediated by BMPR1A (Alk5) inducing Smad 2 and 3 phosphorylation and not via the traditional BMP pathway (Mazerbourg *et al.* 2004).

There is also some evidence that Inverdale and Booroola mutations may share a common pathway because heterozygous crossbred ewes show that the effect of the two genes

in combination is higher than the sum of the effects of each gene alone (Davis *et al.* 1999). This notion is corroborated by recent studies with rat granulosa cells and transfected human cells showing that BMP 15 signals through BMPRII and BMPRII and, after ligand-binding, triggers Smad 1 phosphorylation. However, BMP 15 binds with high affinity to BMPRII and then recruits the BMPRII in opposition to other BMPs that have low affinity for either receptor individually. Moreover, the effect of BMP 15 on steroidogenesis is mediated by the BMP receptors, whereas its effect on granulosa cell proliferation seems to be mediated by MAP kinase (Moore *et al.* 2003). An interesting question arises from the BMP 15 and GDF9 heterodimers, since each homodimer has a higher affinity for a different combination of receptors and uses alternative intracellular receptor-regulated Smad pathways.

Use of prolific sheep in commercial flocks

The impact of the introduction of highly prolific ewes on commercial flocks remains an open question and part of the answer is related to the husbandry level, farm type, lamb price and whether the objective of production is milk, wool or meat. In a review comparing the performance of Booroola crosses with different breeds in different countries in Europe, Oceania and Africa, ewe carriers of the Q249R mutation showed the highest productivity (total weight of lamb produced per ewe joined). However, productivity was below expectations for the reproductive rate owing to high lamb mortality and light lamb weights at weaning (Davis *et al.* 1991). This is also the case under South American environmental and husbandry conditions in Uruguay and Brazil (Villaroel *et al.* 1990; Fernandez Abella 1991).

Higher lamb mortality in carriers is more prevalent in litters with three or more lambs, and occurs within a few hours of parturition. This deleterious side effect of increased prolificacy can be alleviated if the mutation is introduced into breeds with good mothering ability and of large mature size to increase lamb birthweight, and is allied to close supervision at parturition (Davis and Hinch 1985).

Lighter weight lambs at weaning contributed to the reduction in productivity of Booroola-crossed flocks and were generally thought to be intrinsic to the larger litter size and lighter birthweight (Davis *et al.* 1991). Recently, a quantitative trait locus (QTL) for low weaning weight has been suggested to be segregating together with the Booroola mutation; the putative locus is ~20 centimorgans distal from the BMPRII gene (Walling *et al.* 2000). If the QTL is confirmed, there is scope for selection to overcome, at least partially, the lower lamb weight at weaning in prolific flocks, thus reducing the finishing time.

In prolific sheep breeds raised in small flocks, mainly for subsistence purposes, such as the Garole and Javanese Thin Tail (Roberts 2000; Davis *et al.* 2002), the Q249R mutation

has survived almost without the benefit of selection and no aid from ovulation rate records and progeny tests. More intensive management of these ewes may be required in order to take full advantage of the increase in reproductive rate resulting from the mutation.

Economic analyses of the use of prolific sheep have been undertaken after the introgression of the Booroola and Inverdale mutations. The economic value of prolificacy varied highly across farm type with those in better environmental conditions and with more intense husbandry profiting more from the increased ovulation rate. Under the farming conditions of New Zealand, the introduction of one allele of either mutation resulted in increased profitability ranging from \$4.69 to \$ 16.23 per ewe lambing. The additional value of a second Booroola allele was small or negative. The key factors influencing economic output were base-flock prolificacy, lamb survival rate and the slaughter of lambs at a fixed age or weight (Amer *et al.* 1999). Another economic study was conducted to evaluate the increase in prolificity by introducing the Booroola mutation in Assaf and Awassi breeds of dairy sheep in Israel, in a milk production system where 40% of the income came from lamb sales (Gootwine *et al.* 2001). Crossing the dairy ewes with Booroola homozygous Merino rams drastically reduced milk production, with the effect being noticeable even in the subsequent backcrosses. Milk production only became similar to the Awassi breed from the third backcross onwards. The introgression of one allele of the Booroola mutation produced a deficit under most conditions and only when a second allele was introduced by a second backcross was the project marginally profitable. In the more productive Assaf breed, the introgression always produced a deficit. However, the price of genotyping has an impact on the cost of introgression and, perhaps with a genetic test now being available, the profitability of the introduction could be improved. To achieve a profit in dairy flocks, homozygous rams from the third and fourth backcrosses have to be used to introduce the mutation in Awassi and Assaf ewes respectively (Gootwine *et al.* 2001).

Conclusions

The gene mutations that affect ovulation rate in sheep undoubtedly provide an excellent animal model to investigate the mechanisms influencing follicle selection and ovulation rate. It is now clear that the BMP family is one of the factors, if not the major factor, regulating ovulation rate and prolificacy in sheep. The mechanism by which these mutated genes alter the growth and number of ovulatory follicles is not fully understood. However, their use could be an alternative to increase sheep production, although this depends on the management of the production systems and prevailing environmental and economic conditions. In addition, careful analysis of the economic impact of the increased prolificacy has to be conducted before the widespread use of these

mutations could be recommended for commercial flocks. Nevertheless, the arrival of new genetic tests that quickly identify genotypes will help to speed up research on prolific sheep breeds and could become useful tools for farm management in the future.

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