

The oocyte and its role in regulating ovulation rate: a new paradigm in reproductive biology

K P McNatty, L G Moore, N L Hudson, L D Quirke, S B Lawrence, K Reader, J P Hanrahan¹, P Smith, N P Groome², M Laitinen³, O Ritvos³ and J L Juengel

AgResearch, Wallaceville Animal Research Centre, PO Box 40063, Upper Hutt, New Zealand, ¹Teagasc, Athenry Research Centre, Athenry, Ireland, ²School of BMS, Oxford Brookes University, Gypsy Lane, Headington, Oxford X3 0BP, UK and ³Biomedicum Helsinki, PO Box 63 (Haartmaninkatu 8), FIN-00014, University of Helsinki, Helsinki, Finland

Correspondence should be addressed to Ken P McNatty; Email: ken.mcnatty@agresearch.co.nz

Abstract

Ovulation rate in mammals is determined by a complex exchange of hormonal signals between the pituitary gland and the ovary and by a localised exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells. From examination of inherited patterns of ovulation rate in sheep, point mutations have been identified in two oocyte-expressed genes, BMP15 (GDF9B) and GDF9. Animals heterozygous for any of these mutations have higher ovulation rates (that is, + 0.8–3) than wild-type contemporaries, whereas those homozygous for each of these mutations are sterile with ovarian follicular development disrupted during the preantral growth stages. Both GDF9 and BMP15 proteins are present in follicular fluid, indicating that they are secreted products. *In vitro* studies show that granulosa and/or cumulus cells are an important target for both growth factors. Multiple immunisations of sheep with BMP15 or GDF9 peptide protein conjugates show that both growth factors are essential for normal follicular growth and the maturation of preovulatory follicles. Short-term (that is, primary and booster) immunisation with a GDF9 or BMP15 peptide-protein conjugate has been shown to enhance ovulation rate and lamb production. In summary, recent studies of genetic mutations in sheep highlight the importance of oocyte-secreted factors in regulating ovulation rate, and these discoveries may help to explain why some mammals have a predisposition to produce two or more offspring rather than one.

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Introduction

Ovulation rate in mammals is determined by a complex exchange of hormone signals between the pituitary gland and the ovary, and by a localised exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells (Dong *et al.* 1996, Galloway *et al.* 2000, Eppig 2001, Richards 2001, Findlay 2003).

From examination of inherited patterns of ovulation rate in sheep, several breeds have been identified with mutations in two growth factor genes that are expressed in oocytes, namely, bone morphogenetic protein 15 (BMP15), also known as growth differentiation factor 9B (GDF9B), and GDF9 (see McNatty *et al.* 2003 for review). BMP15 and GDF9 are two closely related members of the transforming growth factor- β (TGF β) superfamily, many of which are important for regulating ovarian follicular development (Chang *et al.* 2002, Knight & Glister 2003, Lin *et al.* 2003, Shimasaki *et al.* 2004). However, what distinguishes BMP15 and GDF9 from other TGF β

superfamily members is that changing concentrations of these two factors *in vivo* leads to incremental changes in ovulation rate in sheep (Galloway *et al.* 2000, Juengel *et al.* 2002, 2003, McNatty *et al.* 2003, Hanrahan *et al.* 2004). The significance of these discoveries is that the oocyte appears to regulate the growth and differentiation of adjacent somatic cells as well as their responsiveness to endocrine signals and thereby the number of follicles that mature and ovulate.

The purpose of this review is to summarise recent results showing that BMP15 and GDF9 are important factors regulating ovulation rate. Particular emphasis will be placed on the BMP15 and GDF9 mutations and physiological results arising from studies in sheep.

Some functional and structural properties of BMP15 and GDF9

Within the ovary, both BMP15 and GDF9 mRNA and protein are localised exclusively to the oocyte. The only

exception to this is that GDF9 mRNA and protein have been localised to granulosa cells immediately adjacent to the oocyte in some primates (Sidis *et al.* 1998, Duffy 2003). It is also worth noting that BMP15 and GDF9 mRNA have been identified in the pituitary glands and testes of some species, albeit in limited amounts (Fitzpatrick *et al.* 1998, Otsuka & Shimasaki 2002), suggesting that these factors have some regulatory roles in other tissues.

In sheep, BMP15 mRNA and protein are not present until follicles have just begun to grow (that is, the primary stage of development): thereafter, BMP15 is localised to oocytes of most, if not all, developing follicles (Galloway *et al.* 2000, Juengel *et al.* 2002). GDF9 mRNA and protein can be identified in sheep oocytes during follicular formation, in primordial (type 1) follicles and in oocytes at all stages of follicular growth (Bodensteiner *et al.* 1999, Juengel *et al.* 2002, Mandon-Pepin *et al.* 2003). Under *in vitro* conditions, GDF9 protein is present in both normal-looking and degenerating ovine oocytes, and even in highly disorganised follicular structures, indicating that its distribution is widespread in oocytes notwithstanding the functional status of its surrounding somatic cells (unpublished data). There are species differences with respect to the ontogeny of expression of both BMP15 and GDF9 during follicular development (Aaltonen *et al.* 1999, Bodensteiner *et al.* 1999, Eckery *et al.* 2002, Erickson & Shimasaki 2003).

Like other TGF β superfamily members, BMP15 and GDF9 are translated as preproteins composed of a signal peptide, a large proregion and a smaller mature region (Fig. 1). After removal of the signal peptide, further intracellular processing results in the separation of the biologically active mature region from the proregion (Kingsley 1994). Under reducing conditions, both the mature and unprocessed promature forms of BMP15 and GDF9 can be identified in follicular fluid from sheep (unpublished data), and it remains to be determined what are the final forms of these proteins that regulate target cell functions. Normally, the biologically active form of a TGF β superfamily member is a covalently linked homo- or heterodimer (Fig. 1). However, BMP15 and GDF9 lack the fourth

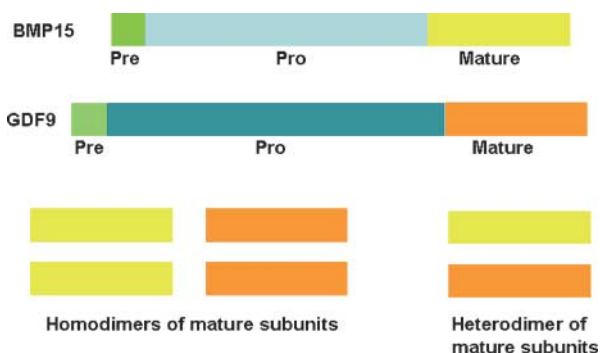


Figure 1 Schematic outline of the BMP15 and GDF9 molecules.

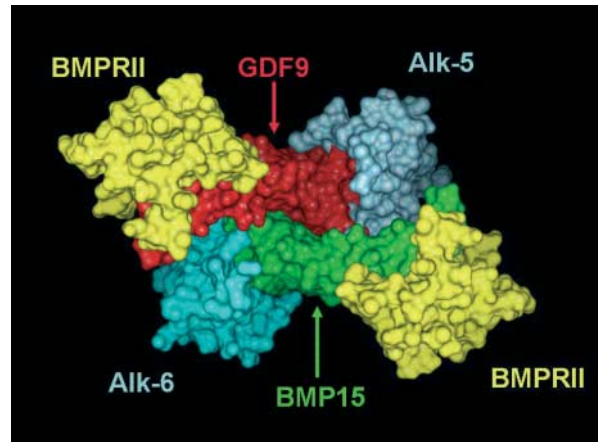


Figure 2 Molecular surface representation of a putative heterodimer (BMP15/GDF9) bound to the extracellular domains of two type I receptors (ALK5) and two type II receptors (BMPRII). Arrows indicate the BMP15 (green) and GDF9 (red) molecules, whereas ALK5 and ALK6 are coloured light and dark blue respectively and BMPRII coloured yellow. The non-covalently linked GDF9 and BMP15 molecules can be compared with the palms of each hand in contact with one another, and the BMPRII receptors attached to the knuckles and the ALK5 and ALK6 receptors to the fingertips.

of the normally conserved seven cysteines that are required for the intersubunit disulphide bridge (McPherron & Lee 1993, Laitinen *et al.* 1998). *In vitro* studies using cell expression systems indicate that both BMP15 and GDF9 can be expressed into media as non-covalently linked homodimers, or coexpressed as heterodimers (Liao *et al.* 2003).

The interaction of BMP15 or GDF9 with the target cell is thought to be via two types of membrane-bound serine/threonine kinases, of which there are seven type I receptors (ALK1-7) and five known mammalian type II receptors (Act R-II, Act R-IIB, AMHR-II, BMPR-II and TGF β R-II). BMP15 is reported to bind to a BMP type I receptor (ALK6) and to BMPR-II (Moore *et al.* 2003), whereas GDF9 is reported to bind to a TGF β type 1 receptor (ALK-5) (Mazerbourg *et al.* 2004) and to BMPR-II (Vitt *et al.* 2002). An example of how a dimeric or heterodimeric BMP15 or GDF9 binds with the extracellular domains of a TGF β type I and BMPR-II receptor is shown in Fig. 2. In the sheep ovary, mRNA and protein for ALK5, ALK6 and BMPR-II are observed in both granulosa cells and oocytes of preantral follicles, indicating that both GDF9 and BMP15 are likely to influence early follicular development (Wilson *et al.* 2001, Souza *et al.* 2002, unpublished data). The nature of the interactions between homodimers of BMP15 or GDF9 or putative BMP15/GDF9 heterodimers and type I and II receptors are still unresolved, but the downstream signalling pathway involves activation of the Smad proteins (Heldin *et al.* 1997, Miyazono 2000, Kloos *et al.* 2002). The effects of BMP15 or GDF9 on follicular cell DNA or steroid synthesis remain to be resolved but appear to differ across species (see Shimasaki *et al.* (2004) for a recent review).

BMP15 and GDF9 mutations in sheep and their effects on ovulation rate

Sheep BMP15 maps to the X chromosome, and the full-length coding sequence of 1179 nucleotides encodes a preproprotein region of 268 amino-acid residues and a mature protein region of 125 amino-acid residues (Galloway *et al.* 2000). Sheep GDF9 maps to chromosome 5 (Sadighi *et al.* 2002), and the full-length coding sequence of 1359 nucleotides encodes a preproprotein region of 318 amino-acid residues and a mature protein region of 135 amino-acid residues (Fig. 1) (Bodensteiner *et al.* 1999).

Currently, five different point mutations have been identified in the BMP15 gene and one in the GDF9 gene, each having a major effect on ovulation rate (Galloway *et al.* 2000, Bodin *et al.* 2003, Hanrahan *et al.* 2004). These point mutations in BMP15 and GDF9 and the resulting amino-acid changes are summarised in Table 1. All animals heterozygous for any one of these BMP15 or GDF9 mutations have increased ovulation rates, whereas homozygous individuals are sterile with normal ovarian follicular development arrested or abnormal from the type 2 stage of growth (Braw-Tal *et al.* 1993, Galloway *et al.* 2000, Bodin *et al.* 2003, Hanrahan *et al.* 2004, unpublished data). From *in vitro* studies, using a cell line expressing recombinant human BMP15 with the FecX^L or FecX^B sheep mutation or recombinant human GDF9 with the sheep FecG^H mutation (Table 1), it was found that the secretion levels of both proteins were significantly lower than those in the wild-types (Liao *et al.* 2004). Surprisingly, equivalent concentrations of the homozygous mutant forms of BMP15 or GDF9 protein had similar levels of biological activity to that of the wild-type proteins when assessed by a thymidine incorporation assay using rat granulosa cells. The implication of these findings is that sheep with the homozygous mutations FecX^B, FecG^H or FecX^L would have biologically active BMP15 or GDF9, although not in sufficient amounts to support ovarian follicular growth since the ovarian phenotypes in these

animals are similar to those in animals having the FecX^H or FecX^G stop codons and thus, presumably, no biologically active protein.

The mean ovulation rates of F700 Belclare, Cambridge, Hanna and Inverdale ewes heterozygous for the known BMP15 and GDF9 mutations, together with the effects of the mutations on the BMP15 or GDF9 protein or their possible interactions with a type I or II binding domain, are summarised in Table 2. Animals with a likely 50% reduction in intrafollicular concentrations of BMP15 (FecX^G, FecX^H or FecX^L) have a mean ovulation rate increase of 35–67%, whereas animals heterozygous for a mutation in the region of BMP15 or GDF9 that interacts with a type I or II receptor (FecX^B or FecG^H) have mean ovulation rate increases of 87–95%. Of interest are the animals that are heterozygous for mutations in both BMP15 and GDF9 (such as FecX^G and FecG^H, and FecX^B and FecG^H). These animals are likely to have reduced concentrations of BMP15 and possibly an altered interaction between GDF9 and its type I receptor (such as FecX^G and FecG^H) or an altered interaction for both BMP15 and GDF9 with their receptor-binding domains (FecX^B and FecG^H). The consequence of these double mutations appears to be a synergistic effect on ovulation rate (Table 2). The mean ovulation rates in Lacaune ewes, heterozygous for the BMP15 mutation leading to a tyrosine substituting for a cysteine at mature peptide residue 53 (FecX^L; Table 1), is not known, as carrier animals currently identified have another co-dominant mutation on chromosome 11 (Bodin *et al.* 2003).

The evidence from gene-knockout studies in mice suggests that BMP15 and GDF9 may not affect follicular development and ovulation rate in a similar manner in all species. Female mice lacking a functional GDF9 gene are infertile and have an ovarian phenotype similar to that described for sheep homozygous for the BMP15 (FecX^G, FecX^B, FecX^H, FecX^L and FecX^L) or the GDF9 mutations (FecG^H) (Table 1) (Braw-Tal *et al.* 1993, Dong *et al.* 1996, Galloway *et al.* 2000, unpublished data). However, mice lacking a functional BMP15 gene have apparently normal follicular development and are fertile, although litter size is reduced by impaired fertilisation of oocytes (Yan *et al.* 2001). Mice heterozygous for an inactive copy of the BMP15 or GDF9 gene appear to have normal litter sizes, whereas those heterozygous for inactive copies of both a BMP15 and a GDF9 gene have reduced litter sizes: this effect was enhanced in mice without a functional BMP15 gene and heterozygous for an inactive GDF9 gene. In all cases, it seems that follicular growth was normal but that fertilisation of the oocyte was seriously impaired. The reason for the reduced litter size was thought to be inappropriate development of the oocyte–cumulus cell complex, as many oocytes were recovered with few, if any, attached cumulus cells (Yan *et al.* 2001).

Table 1 Polymorphic sequence variations in BMP15 and GDF9 that affect ovarian follicular development and ovulation rate in sheep.

Gene	Allele	Base change	Coding residue (aa)	Mature peptide residue (aa)	Amino-acid change
BMP15	FecX ^G	C → T	239	–	Gln → STOP
	FecX ^B	G → T	367	99	Ser → Ile
	FecX ^L	T → A	299	31	Val → Asp
	FecX ^H	C → T	291	23	Glu → STOP
	FecX ^L	G → A	321	53	Cys → Tyr
GDF9	FecG ^H	C → T	395	77	Ser → Phe

Data from Galloway *et al.* (2000), Bodin *et al.* (2003) and Hanrahan *et al.* (2003). For the purposes of this report, the BMP 15 mutation in Lacaune sheep was assigned the allele FecX^L. This mutation was found as a co-dominant mutation in a Lacaune breed with an autosomal gene. aa = amino acid.

Table 2 Mean ovulation rate (and percentage increase above WT) in various sheep breeds/lines.

Wild-type, mutation or immunisation	Predicted effect on BMP15, GDF9 or receptor type	Hanna ^a	Inverdale ^a	F700 Belclare ^b	Cambridge ^b	Romney ^c (immunised)
WT	None	1.8	1.8	1.9	2.3	1.6
FecX ^H (BMP15)	Reduced concentrations of mature BMP15	3.0 (+67%)	–	–	–	–
FecX ^I (BMP15)	Reduced concentrations of mature BMP15	–	2.9 (+61%)	–	–	–
FecX ^G (BMP15)	Reduced concentrations of mature BMP15	–	–	2.7 (+42%)	3.1 (+35%)	–
FecX ^B (BMP15)	Disrupted interaction type II with the receptor-binding domain	–	–	3.7 (+95%)	–	–
FecX ^H (GDF9)	Disrupted interaction with the type I receptor-binding domain	–	–	–	4.3 (+87%)	–
FecX ^G & FecG ^H	Reduced concentrations of mature BMP15 and disrupted interactions with a type I receptor-binding domain	–	–	–	5.8 (+152%)	–
FecX ^B & FecG ^H	Disrupted interactions with the type I and II receptor-binding domains	–	–	6.1 (+221%)	–	–
Immunised (BMP15-peptide 2)	Disrupted interaction with a putative type I receptor-binding domain	–	–	–	–	2.9 (+81%)
Immunised (GDF9 peptide 2)	Disrupted interaction with a putative type I receptor-binding domain	–	–	–	–	3.5 (+121%)

WT = wild-type animals.

^aDavis *et al.* 2001.

^bHanrahan *et al.* 2004.

^cUnpublished data.

Regulation of ovulation rate by altering BMP15 and GDF9 concentrations *in vivo*

The implication of the ovulation rate outcomes in sheep heterozygous for mutations in either BMP15 or GDF9 is that the absence or extremely low concentrations of either of these factors leads to an inhibition of ovarian follicular development and thus sterility, whereas a partial reduction in concentrations of these factors increases ovulation rate and thus litter size (Davis *et al.* 2001, Hanrahan *et al.* 2004). Inverdale ewes heterozygous for the BMP15 mutation (FecX^I) have similar concentrations of plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to their wild-type contemporaries. Moreover, the ovarian secretions of steroids are not different between the heterozygous and wild-type Inverdales, notwithstanding the fact that in heterozygotes the mean ovulation rate is higher and there are more oestrogenic follicles (Shackell *et al.* 1993). The underlying cause of the higher ovulation rate in heterozygous Inverdales appears to be the precocious maturation of small follicles due to increased FSH responsiveness and an earlier acquisition of LH receptors by the granulosa cells (Shackell *et al.* 1993). This is consistent with the *in vitro* studies of Otsuka *et al.* (2001) showing that BMP15 can cause suppression of FSH receptor expression in rat granulosa cells, thereby suggesting that reduced BMP15 concentrations are associated with a higher level of FSH responsiveness. Moreover, the higher ovulation rate in ewes heterozygous for the GDF9 mutation FecG^H is consistent with at least one report

showing that GDF9 inhibits FSH actions in rat granulosa cells *in vitro* by inhibiting FSH-dependent LH receptor expression, cAMP production, oestradiol and progesterone synthesis (Vitt *et al.* 2000).

Since the oocyte is a major source of both BMP15 and GDF9 and the major target for these factors is the granulosa cells (see Shimasaki *et al.* 2004 for a review), it is reasonable to infer that these oocyte-derived growth factors modulate the responsiveness of granulosa cells to the gonadotrophins. We therefore asked whether the intrafollicular concentrations of BMP15 or GDF9 could be modulated by exogenous means to alter ovulation rate. We have addressed this question, using sheep as our experimental model, and the results from such studies have been very informative.

Long-term immunisations with BMP15 or GDF9 peptides

In one study, ewes were immunised with either keyhole limpet haemocyanin (KLH) or KLH conjugated to either a 15 mer BMP15 or a GDF9 peptide sequence from the N-terminal end of each mature protein region (that is, BMP15 peptide 1 or GDF9 peptide 1) (see Juengel *et al.* 2002). The immunisations, which took place before and during the breeding season, were given i.m. a month apart in Freund's adjuvant for 7 months. Ewes immunised with KLH alone (9/9) maintained regular oestrous cycles of 17 ± 2 days during the breeding season. By contrast, most ewes immunised against BMP15 (9/10) or GDF9 (10/10)

failed to show normal oestrous behaviour throughout the breeding season. Similarly, most of the BMP15- and GDF9-immunised ewes showed no evidence of ovulation or normal patterns of luteal progesterone concentrations. When the ovaries were examined at the end of the experiment (7.5 months after the initial immunisation), it was evident that normal follicular development beyond the type 2 stage of growth did not occur in either the BMP15- or GDF9-immunised animals and that the phenotype was similar to that observed in ewes homozygous for mutations in BMP15 or GDF9 (Braw-Tal *et al.* 1993, Galloway *et al.* 2000, Hanrahan *et al.* 2004, unpublished data). As shown by ELISA, ewes immunised against the BMP15 peptide had high antibody titres against ovine (o) BMP15 but showed no cross-reactivity (that is, <1%) to oGDF9. Likewise, ewes immunised with the GDF9 peptide contained antibodies against oGDF9 protein, but not against oBMP15 protein. These data show that secreted forms of both BMP15 and GDF9 are essential for follicular development and ovulation rate in sheep. The aforementioned BMP15 and GDF9 amino-acid sequences (that is, peptide 1) correspond to flexible regions of the respective mature protein regions, and it is not known whether these regions are close to a binding site or a region important for dimerisation. To evaluate the affect of immunising sheep against alternative peptide sequences, we conjugated a 16 mer BMP15 peptide sequence (BMP15, peptide 2) or a 15 mer GDF9 peptide sequence (GDF9, peptide 2), near a putative type 1 receptor-binding region to KLH. Ewes ($n = 9-10$ per group), including a KLH control group, were immunised at monthly intervals in Freund's adjuvant, starting in the non-breeding season and continuing through the breeding season (six monthly injections). The ewes in all treatment groups underwent normal cyclical oestrous activity, and there was no evidence either at laparoscopy (on two separate occasions) or at ovary recovery that any of these treatments caused a reduction in ovarian follicular development. Indeed, the mean \pm S.E.M. ovulation rates were, on most occasions, significantly higher in both the BMP15- and GDF9-treated groups than in the KLH control group (Table 3). When the antibody responses were tested by ELISA, all the BMP15 and GDF9 animals produced antibody responses to BMP15 and GDF9 respectively, but none of the KLH

Table 3 Mean ovulation rates in ewes following long-term immunisation with KLH, KLH-GDF9 peptide 2, or KLH-BMP15 peptide 2.

Treatment	Mean ovulation rate (\pm S.E.M.)	N
KLH	1.6 \pm 0.2 ^a	10
KLH-GDF9 (2)	3.6 \pm 0.5 ^b	10
KLH-BMP15 (2)	3.1 \pm 0.4 ^b	9

N = number of ewes. Values with different superscripts are significantly different, $P < 0.01$. Unpublished data. GDF9 peptide 2 and BMP 15 peptide 2 refer to 15 or 16 mer peptides near a putative BMP type 1 receptor-binding region.

control immunised animals produced antibodies to either BMP15 or GDF9. The overall mean ovulation rate increases following the BMP15 (peptide 2) or GDF9 (peptide 2) immunisations were similar to those observed for the heterozygous sheep (FecX^B and FecG^H), where there were mutations in the regions of BMP15 or GDF9 that interact with the type I or II receptors (see Table 2).

Short-term treatments with BMP15 or GDF9 antibodies

Short-term passive immunisation studies using ovine antiplasma against BMP15 (peptide 1) or GDF9 (peptide 1) indicated that both BMP15 and GDF9 are important for the final phases of follicular maturation (Juengel *et al.* 2002). This conclusion was based on the following experimental results: four of the five ewes receiving antiplasma against BMP15 failed to ovulate, and three of the five ewes were devoid of surface visible follicles. All ewes ($n = 5$) receiving antiplasma against the GDF9 peptide formed one or two corpora lutea, but three of five ewes displayed abnormal luteal phase patterns of progesterone concentrations. All ewes ($n = 4$) receiving KLH antiplasma ovulated with normal luteal phase patterns of progesterone concentration.

Short-term immunisations with BMP15 or GDF9 peptides

A number of BMP15 and GDF9 peptide formulations have been tested for their ability to increase ovulation, fertilisation or lambing rates (Juengel *et al.* 2003, McNatty *et al.* 2003, unpublished data). The concept being tested is whether it is possible to mimic reliably the lambing and/or ovulation rate increases that are found in ewes that have heterozygous mutations in either BMP15 or GDF9 (Davis *et al.* 2001, Hanrahan *et al.* 2003) (Table 1). Using a primary and single booster vaccination with BMP15 (peptide 1) or GDF9 (peptide 1) conjugated to bovine serum albumin (BSA) in a water-based adjuvant, Juengel *et al.* (2003) reported consistent and significant increases in ovulation rate in ewes immunised with GDF9 peptide (+22%; $P < 0.05$ (χ^2 analysis); $n = 30$ animals) or BMP15 peptide (+44%; $P < 0.05$ (χ^2 analysis); $n = 30$ animals) compared with BSA-immunised controls ($n = 50$ animals). Importantly, no adverse affects were observed on fertilisation, embryo survival or ability of ewes to maintain their pregnancy.

A larger study, using BMP15 peptide 1 conjugated to a carrier protein ($n = 94$ animals) or carrier protein alone ($n = 49$ animals) with the same water-based adjuvant as described above (see Juengel *et al.* 2003), recorded a 25% increase in both ovulation and lambing rate in the BMP15-immunised group relative to the carrier protein control group (both $P < 0.05$ (student's t-test); unpublished data).

Possible molecular forms of BMP15 and GDF9 in regulating ovulation rate in sheep

GDF9 and BMP15 potentially could function as homodimers, heterodimers, a combination of the two or some other configuration. We have examined a number of possible relationships between ovulation rate and GDF9 and BMP15 acting as either homodimers or heterodimers. The ovulation rate data considered are those shown in Table 1 for sheep heterozygous for the FecX^I, FecX^H, FecX^G, FecX^B, FecG^H, FecX^G and FecG^H, or FecX^B and FecG^H mutations. The assumptions that were made for GDF9 and BMP15 functioning as homodimers were as follows: (1) the BMP mutations generating stop codons (FecX^H and FecX^G) (Tables 1 and 2) or the Inverdale (FecX^I) variant of BMP15 (Tables 1 and 2) reduces biological activity at the level of the receptor(s) to 0.5 times that of the wild-type; (2) the mutations in the regions of BMP15 or GDF9 that interact with the type I or II receptors (FecX^B and FecG^H) (Tables 1 and 2) reduce biological activity at the level of the receptor to 0.25 times that of the wild-type; and (3) mutations in both BMP15 and GDF9 are multiplicative, reducing biological activity at the level at the receptor(s) to 0.125 (FecX^G and FecG^H; 0.5×0.25) and 0.0625 (FecX^B and FecG^H; 0.25×0.25) times that of the wild-types. The assumptions made for FecX^H or FecX^G having only 50% of normal biological activity was based on the knowledge that these heterozygous animals would have only 50% of normal concentrations of mature BMP15 because only half of the normal amount of protein could be produced. Animals with the FecX^I mutation were also given the figure of 50% of normal BMP15 activity on the assumption that the FecX^I-produced BMP15 would not dimerise avidly with normal BMP15 protein (Galloway *et al.* 2000). For the FecX^B or FecG^H mutations which require unmutated dimers for normal receptor function, the assumptions were based on the allelic frequency of normal biological activity being 25% of the wild-type because only 25% of homodimers in heterozygous animals consist of unmutated proteins. The multiplicative activities were derived from multiplying FecX^G and FecG^H (that is, 0.5×0.25) or FecX^B and FecG^H (that is, 0.25×0.25). Consequently, if BMP15 and GDF9 function as homodimers, the relationship between ovulation rate (y) and theoretical activation level of the receptor (x) as a fraction of the wild-type can be expressed by the equation $y = -77 \ln(x) + 91$ ($R^2 = 0.96$; $P < 0.001$; regression analysis).

The assumptions made for GDF9 and BMP15 functioning as heterodimers were based on the reasoning outlined above, namely: (1) that the BMP mutations generating stop codons (FecX^H and FecX^G), the Inverdale variant (FecX^I) or those interfering with type I or II receptor binding (FecX^B and FecG^H) reduce biological activity at the level of the receptor(s) to 0.5 times that of the wild-type; and (2) mutations in two of the above genes are multiplicative, namely, 0.25 times that of the wild-type. The assumptions made for FecX^H, FecX^G and FecX^I were

the same as those described for the homodimers. For FecX^B and FecG^H, the assumptions were based on the allelic frequency of normal biological activity being 50% of the wild-type because 50% of the heterodimers in heterozygous animals consist of unmutated proteins. With these assumptions, the relationship between ovulation rate (y) and theoretical activation level of the receptor as a fraction of the wild-type (x) can be expressed by the equation $y = 146 \ln(x) + 68$ ($R^2 = 0.82$; $P < 0.001$; regression analysis).

From these associations, the preferred interpretation is that GDF9 and BMP15 affect follicular development and ovulation rate in sheep as functional homodimers. Verification of this hypothesis will require further *in vivo* and *in vitro* testing. It is important to note that the homodimer hypothesis does not exclude the notion of interactions between GDF9 and BMP15 either at the level of receptor or during the post-receptor signalling pathway. Moreover, it does not exclude the possibility that two heterodimers are necessary to activate a type I and II receptor complex.

Conclusion

Recent studies of genetic mutations in sheep highlight the importance of oocyte-secreted factors in regulating ovarian follicular development and ovulation rate. These findings, together with the immunisation results with BMP15 and GDF9 peptides, demonstrate that by altering the bioavailability of GDF9 and BMP15 *in vivo*, it is possible by exogenous means to enhance ovulation rate and increase lamb production or to induce infertility. Future studies with GDF9 and/or BMP15 peptides are aimed at translating these findings in sheep to regulating ovulation rate and numbers of offspring in other farmed animals, primates, wildlife or endangered species. The role of oocyte-derived growth factors in either up- or downregulating fertility is an exciting new paradigm in reproduction biology.

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