Using sheep lines with mutations in single genes to better understand ovarian function

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Abstract

Livestock populations have been subjected to strong selection pressure to improve reproductive success, and this has led to the identification of lines of animals with increased fecundity. These animals provide a rich biological resource for discovery of genes and regulatory mechanisms that underpin improved reproductive success. To date, three genes, all related to the transforming growth factor β pathway, have been identified as having mutations that lead to alterations in ovulation in sheep. In addition, several other sheep lines have been identified with putative mutations in single genes with major effects on ovulation rate. This review is focused on the identification of the mutations affecting ovulation rate and how these discoveries have provided new insights into control of ovarian function.

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Genetic mutations affecting ovulation rate in sheep

Multiple mutations in three different genes, all related to the transforming growth factor β (TGFB) superfamily pathway, have been identified in various sheep lines around the world (Table 1). Sheep lines with other putative major genes have also been established, some of which do not appear to interact with the TGFB superfamily and thus may be affecting other pathways yet to be discovered.

BMP15 mutations

At least six different mutations have been identified in the *BMP15* gene (Galloway *et al.* 2000, Hanrahan *et al.* 2004, Bodin *et al.* 2007, Martinez-Royo *et al.* 2008, Monteagudo *et al.* 2009, Lahoz *et al.* 2011) wherein ewes heterozygous for the mutations have increased ovulation rates and those homozygous for the mutations are infertile. While all the mutations have similar general effects on fertility, there are subtle differences, as the increases in ovulation rate observed in the heterozygous animals (Table 1) vary from 35 to 100% (McNatty *et al.* 2004). While this could be related to differences in the background genetics of the various sheep lines, it has also been hypothesized to be the result of different effects of the mutations on protein function. Those mutations potentially affecting interaction of BMP15 with its receptors are suggested to have greater effects on

ovulation rate given the complex nature of the interactions between BMP15 and GDF9 with their receptors (McNatty *et al.* 2004). Recently, two new mutations in *BMP15* have been described in the French Grivette and the Polish Olkuska breeds. These lead to hyper prolificacy, particularly in ewes homozygous for the mutation, without causing sterility (Demars *et al.* 2012). Based on the known physiological activities of the proteins, this phenotype would be predicted if the mutation decreased, rather than abolished, the biological activity of the protein (Fig. 1).

GDF9 mutations

Studies on mice showed that GDF9 (Dong *et al.* 1996), but not BMP15 (Yan *et al.* 2001), was essential for normal follicular growth. Therefore, the question arose as to whether one, but not the other, of these two proteins would be important for normal follicular development, with the key player showing species-dependent activity. Related to this was the key question of whether GDF9 would also be essential for normal follicular growth in sheep. The first indications that GDF9 was indeed essential for normal follicular development in sheep came through active immunization studies. Adult sheep immunized against GDF9 ceased having reproductive cycles with ovarian follicular development affected from the earliest stages (Juengel *et al.* 2002). Shortly after these findings were published, a mutation in *GDF9* was

Table 1 Known mutations affecting *BMP15*, *GDF9* and *BMPR1B* genes, the resulting effect on the encoded proteins and the effects on ovulation rate of ewes heterozygous and homozygous for the mutations.

Gene	Line/breed	Allele	Coding residue (aa)	Amino acid change	Predicted effect	Change in ovulation rate	
						Heterozygous	Homozygous
<i>BMP15</i>	Bel/Cam	<i>FecX^G</i>	239	Gln-STOP	No mature region made	+35–42%	POF
	Belclare	<i>FecX^B</i>	367	Ser-Ile	Disrupt binding to type 2 receptor	+95%	POF
	Inverdale	<i>FecX^I</i>	299	Val-Asp	Reduced concentration of mature BMP15	+61%	POF
	Hanna	<i>FecX^H</i>	291	Glu-STOP	Truncated non-functional protein	+67%	POF
	Lacaune	<i>FecX^L</i>	321	Cys-Tyr	Reduced conc. of BMP15	+100%	POF
	Rasa Arag	<i>FecX^R</i>	154	Frame shift	No mature region made	+38–46%	POF
<i>GDF9</i>	Bel/Cam	<i>FecG^H</i>	395	Ser-Phe	Disrupted binding to type 1 receptor	+87%	POF
	Thoka	Thoka	427	Ser-Arg	Disrupted binding to type 2 receptor	+32% (lambling rate)	POF
	Santa Inês	<i>FecG^E</i>	345	Phe-Cys	Alters dimer formation	+10% (not significant)	+82%
<i>BMPR1B</i>	Booroola	<i>FecB</i>	249	Gln-Arg	Alters intercellular signalling (?)	+86%	+206%

Bel/Cam, Belclare/Cambridge; POF, primary ovarian failure; Rasa Arag, Rasa Aragonesa.

identified in the Belclare and Cambridge breeds in which animals heterozygous for the mutation had increased ovulation rate and those that were homozygous were sterile (Hanrahan *et al.* 2004). This mutation, which introduces a non-conservative amino acid change, is thought to disrupt interaction with a type 1 TGFB family receptor (McNatty *et al.* 2004). Subsequently, a separate mutation in *GDF9* in the Thoka line with a similar, although not identical, phenotype was identified (Nicol *et al.* 2009). The mutation in the Thoka line was predicted to disrupt binding to the type 2 receptor (McNatty *et al.* 2004, Juengel *et al.* 2005). Further, a mutation in *GDF9* has been linked to increased ovulation rate when ewes are homozygous for the non-conservative change in an amino acid predicted to be involved in dimer formation. No change in ovulation rate was observed in heterozygous ewes (Silva *et al.* 2011). This phenotype is similar to that described for the French Grivette sheep with mutations in *BMP15*. Based on the known physiological activities of the proteins, this phenotype would be predicted if the mutation decreased, rather than abolished, the biological activity of the protein. Similarly, a single-strand conformation polymorphism has been identified in the encoded proregion of the *GDF9* gene of the Small Tailed Han sheep with genotypes of AA and AB (Chu *et al.* 2004). The AA genotype had a larger litter size than the AB genotype, suggestive of higher ovulation rates in the homozygous vs the heterozygous carriers. However, interpretation of these data sets is complicated by the lack of any animals with the BB genotype, potentially due to the rarity of the B allele, and the knowledge that mutations in both *BMP15* and *BMPR1B* are also present, but not fixed, in the Small Tailed Han sheep (Davis *et al.* 2006b, Chu *et al.* 2007).

BMPR1B mutation

Although a mutation in the *BMPR1B* gene has been identified in several sheep lines selected for increased fecundity (Davis *et al.* 2002, 2006b, Chu *et al.* 2007), the

same mutation (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001) has been observed in each of these lines. In contrast to the relatively restricted expression of the *GDF9* and *BMP15* genes, *BMPR1B* is widely expressed throughout the body and the mutation likely subtly affects multiple tissue types including the pituitary and the adrenal glands (Wilson *et al.* 2001, Souza *et al.* 2003, Xu *et al.* 2010). However, the major effect of this mutation appears to be at the level of the ovary (Campbell *et al.* 2003, Souza *et al.* 2003). The mutation introduces a non-conservative amino acid change in the kinase domain of the protein (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Original studies showed that the mutated receptor, while potentially having higher basal activity, did not show increased receptor activity in response to ligand treatment (Fabre *et al.* 2003). However, others have failed to observe similar effects when the mutation was introduced into the mouse *Bmpr1b* gene (Ho & Bernard 2009), and the effects of the Booroola mutation on the kinase activity of ovine *BMPR1B* have not been verified (Edwards *et al.* 2009). Thus, it is unclear how this mutation affects the receptor to cause changes in ovarian function.

Gene interactions and other as yet unidentified mutations

Several ewes that carry mutations in more than a single gene have been identified and the combination of these genes has a synergistic effect with very high ovulation rates being reached in some animals. For example, the average ovulation rates (average number of eggs released by animals that can ovulate for any one cycle) of ewes heterozygous for mutations in both *GDF9* and *BMP15* was 252–321% of controls (Hanrahan *et al.* 2004) while ewes heterozygous for mutations in *BMP15* and *BMPR1B* had average ovulation rates 258% of controls (Davis *et al.* 1999). Interactions among these genes and other as yet unidentified mutations have also been demonstrated. For example, the Woodlands gene, an unidentified mutation on the X-chromosome (Davis *et al.* 2001),

Figure 1 The roles of GDF9 and BMP15 in the regulation of ovarian follicular development. (A) GDF9 and BMP15 are produced by the oocyte and act through receptors located on the granulosa cells (GC). Both GDF9 and BMP15 utilize a receptor complex composed of two types of serine–threonine kinases. BMPR2 is the type 2 receptor used by both GDF9 and BMP15 with GDF9 using TGFBR1 (ALK5) and BMP15 using BMPR1B (ALK6) as the type 1 receptors. The Booroola mutation is in BMPR1B and this may account for the synergistic interactions between the Booroola and Inverdale (*BMP15*) mutations. Together, GDF9 and BMP15 stimulate proliferation of granulosa cells and inhibit gonadotrophin-induced maturation. (B) Most follicles are present in the ovary as non-growing type 1 (primordial) follicles. Oocytes in these follicles express GDF9 but not BMP15. Growing follicles express both GDF9 and BMP15 in the oocytes and the granulosa cells become gonadotrophin responsive, and first express FSH receptors. Later in development, the granulosa cells express both FSH and LH receptors and continuous development becomes critically dependent on the gonadotrophins. Initially, a number of follicles begin to grow but the granulosa cells of the maturing follicles produce increasing amounts of steroid and inhibin that lead to inhibition of FSH production from the pituitary gland. Thereafter, only those follicles that have developed LH receptors in the granulosa cells continue to develop when the FSH concentration declines. In wild-type ewes, typically only one or two of the developing antral follicles reach this critical maturational stage when the FSH concentration has declined during the preovulatory period. In turn, this then restricts the number of follicles ovulating to those with LH-responsive granulosa cells. Ewes homozygous for mutations in either GDF9 or BMP15 fail to make sufficient quantities of biologically active protein to stimulate granulosa cell proliferation and thus normal follicular growth is prevented from the type 2 (primary) stage of development. Granulosa cells in ewes that are heterozygous for mutations in either *GDF9* or *BMP15*, or heterozygous or homozygous for mutations in *BMPR1B*, most likely have reduced secretions of GDF9 and/or BMP15 or an attenuated GDF9 and/or BMP15 response. It is proposed that this results in reduced proliferation but an earlier acquisition of LH receptivity in the granulosa cells in follicles at a smaller size. The concentrations of steroids and inhibin are related to the number of granulosa cells in developing follicles. As more follicles have developed LH receptors while FSH concentrations decline, then more follicles will continue to grow during the preovulatory period, thereby leading to higher ovulation rates in these genotypes.

appears to act synergistically with the Inverdale and Booroola genes, with ewes heterozygous for all the three genes having ovulation rates as high as 13 compared with 1–2 for wild-type controls (Davis *et al.* 2008). A possible mechanism for these interactions was identified when it was shown that *BMP15* mRNA levels were decreased in animals carrying the Woodlands gene (Feary *et al.* 2007) or homozygous for the Booroola gene (Crawford *et al.* 2011) compared with their wild-type contemporaries. However, *BMP15* mutations have also been observed in flocks carrying other putative major genes influencing ovulation rate wherein the effect is additive rather than synergistic. This includes the animals carrying the Lacaune gene (*FecL^L*) and the *BMP15* mutation in the Lacaune breed (*FecX^L*) (Drouilhet *et al.* 2009). Investigations into the underlying physiology of ewes homozygous for the Lacaune (*FecL^L*) gene further highlighted the fact that different physiological mechanisms appeared to be acting to increase ovulation rate in this line of ewes (Drouilhet *et al.* 2010). Preliminary publications indicate a mutation in intron 7 of *B4GALNT2*, which causes ectopic expression of the mRNA in the ovarian follicle and an aberrant pattern of glycosylated proteins in granulosa cells (Mansanet *et al.* 2012). The proteins with atypical glycosylation include members of the TGFB superfamily, and thus, the question still does remain whether this mutation interacts with the TGFB pathway. Analysis of another putative gene segregating in the AgResearch Fertility flock, the Davisdale, also indicates an additive effect with the Inverdale mutation (Juengel *et al.* 2011c). Thus, it would not be unexpected to find that pathways other than the TGFB superfamily are likely to also be key regulators of ovulation rate in sheep.

Other sheep lines have been identified with putative single genes affecting ovulation rate. These include the Wishart line (Davis *et al.* 2006a), a potential second gene in the New Zealand Booroola line (Juengel *et al.* 2008) and the Belle-Ile breed (Davis 2004). However, little is known about the underlying physiology that leads to the observed increases in ovulation rate/litter size or whether any of the aforementioned sheep lines have perturbations in the TGFB superfamily pathway. Identifying the mutations underlying these phenotypes, as well as the pathways affected, is likely to offer new insights into the range of physiological factors regulating ovulation rate.

Mechanisms of action of GDF9 and BMP15 and the involvement of the BMBR1B receptor

One complicating factor in understanding the actions of GDF9 and BMP15 is the apparent differences among species in the role of these factors in regulating ovarian follicular development. Considerable knowledge regarding the potential roles of GDF9, BMP15 and BMPR1B has been gained from studying the physiology of ewes

carrying mutations in the genes encoding these proteins (Souza *et al.* 2003, 2004, McNatty *et al.* 2005a, Fabre *et al.* 2006) and this is the focus of this review. However, data from other species will be included when it is unavailable for sheep. Also outside the scope of this review is in-depth discussion of the structure of GDF9 and BMP15, the receptors and signalling pathways activated by GDF9 and BMP15 as well as the potential ligands for BMPR1B, and the functions of GDF9 and BMP15 as assessed *in vitro*. Information on these aspects is reported extensively elsewhere (Shimasaki *et al.* 2004, Juengel & McNatty 2005, Gilchrist *et al.* 2008, Otsuka *et al.* 2011, Paulini & Melo 2011). However, it is important to note that mRNA encoding *GDF9* and *BMP15* are tightly co-regulated in multiple species (Crawford & McNatty 2012) and GDF9 and BMP15 have synergistic effects at the level of the cell (McNatty *et al.* 2005b, 2005c). Thus, when examining the physiology of these molecules, the cooperative nature of these molecules is a very important consideration.

Phenotypically, ewes homozygous for mutations in *GDF9* or *BMP15* are similar, with growth of follicles blocked at a very early stage of development (Braw-Tal *et al.* 1993, Nicol *et al.* 2009). This supports the roles of both GDF9 and BMP15 in proliferation of granulosa cells very early in the growth of the follicle (Fig. 1). In most cases, in ewes lacking biologically active GDF9 or BMP15, normal follicular development beyond the primary stage of development is not observed, albeit there are differences between the *GDF9* mutations with abnormal follicles up to the antral stage observed in some lines (Hanrahan *et al.* 2004, McNatty *et al.* 2005a). An interesting feature observed in ewes lacking either GDF9 or BMP15 is the continued growth of the oocyte to a size that is similar to that seen in an antral follicle (Braw-Tal *et al.* 1993, Juengel *et al.* 2000, Nicol *et al.* 2009). This results in the formation of abnormal follicles with a single layer of flattened granulosa cells surrounding enlarged oocytes, suggesting that at least some of the growth of the oocyte can occur independently of the granulosa cells. Eventually, however the oocytes die, and structures containing granulosa cells and degenerating oocytes are commonly observed. Based on gene expression data, it is thought that the granulosa cells subsequently form 'nodules' containing residual granulosa cells (Juengel *et al.* 2000, Nicol *et al.* 2009).

In ewes lacking active BMP15, development of benign ovarian cysts or tumours occurs (Braw-Tal *et al.* 1993, McLeod *et al.* 1995). These can have different morphologies ranging from a large follicle-like cyst structure, to a semi-solid structure containing granulosa-like cells, to cells with morphology similar to that observed in the corpus luteum, although without an extensive blood supply (Braw-Tal *et al.* 1993, Juengel *et al.* 2000). While these structures have been observed to grow and regress on the ovary throughout the life of the ewe (Braw-Tal *et al.* 1993, McLeod *et al.* 1995),

longevity studies have shown no evidence of metastasis of the tumours over the natural lifetime of these animals. In the limited number of ewes homozygous for the *GDF9* mutation that have been examined, similar structures have not been observed (Nicol *et al.* 2009). The cessation of follicular development at an early stage of development in ewes lacking active GDF9 or BMP15 also disturbs the normal feedback loops between the ovary and hypothalamus and pituitary, and hence, gonadotrophin levels are elevated (Braw-Tal *et al.* 1993, McLeod *et al.* 1995, Nicol *et al.* 2009). The exception to this observation is when benign ovarian cysts or tumours develop as these tumours can produce hormones that affect gonadotrophin secretion (Braw-Tal *et al.* 1993, McLeod *et al.* 1995). The lack of normal ovarian follicular development also affects the development of the uterus. Adult ewes have a reproductive tract with morphology similar to that observed in a young lamb (Davis *et al.* 1992, Nicol *et al.* 2009). Analysis of the uterus of homozygous Inverdale animals showed suppression of uterine development (Hayashi *et al.* 2008). However, it is important to note that the effects on gonadotrophin secretion and uterine development are thought to be indirect due to the absence of normal developing antral follicles.

Ewes heterozygous for mutations in *BMP15* (Inverdale) or homozygous for mutations in *BMPR1B* (Booroola) have also been studied intensively (Fig. 1). As expected, given the known interactions between the Inverdale and Booroola genes (Davis *et al.* 1999), as well as the identification of *BMPR1B* as the receptor for *BMP15* (Moore *et al.* 2003), there are similarities in the changes in physiology observed with these mutations; however, there are also differences. The mRNA encoding *BMP15* is first expressed in the primary (type 2) follicle (Galloway *et al.* 2000), and thus, the effects of *BMP15* are thought to be limited to the growing follicle. In contrast, *BMPR1B* mRNA is expressed in the ovary before and during formation of the follicles and the mutation would appear to affect ovarian follicles from the primordial stage onwards. Differences in follicle and oocyte size and cellular organelles have been observed when comparing primordial follicles from ewes homozygous for the Booroola mutation to their wild-type contemporaries (Reader *et al.* 2012). Whether the numbers of follicles formed are influenced by the Booroola mutation is unclear as different studies have drawn different conclusions, with some studies showing increased numbers of follicles (Smith *et al.* 1994) and others indicating that the number of follicles may be reduced in ewes homozygous for the Booroola mutation (Ruoss *et al.* 2009).

For both the Booroola and the Inverdale animals, a general hypothesis can be developed regarding the mechanisms increasing ovulation rate (Souza *et al.* 2003, Juengel *et al.* 2004b, Fabre *et al.* 2006). In both cases, while the number of ovulating follicles increases, the total number of granulosa cells from the ovulatory

follicles, and thus the total amount of granulosa cell-secreted products such as oestradiol and inhibin, does not change (McNatty *et al.* 1985, Shackell *et al.* 1993, Souza *et al.* 2003, Juengel *et al.* 2004b). This occurs as a consequence of a reduction in the size of the ovulatory follicle and number of granulosa cells present within each follicle. In this manner, endocrine communication between the ovary and the hypothalamus/pituitary remains the same. The follicle therefore develops characteristics of an ovulatory follicle at a smaller size. This can be demonstrated through the increased proportions of smaller diameter follicles in Booroola and Inverdale animals with granulosa cells capable of responding to LH *in vitro* (McNatty *et al.* 2009, Crawford *et al.* 2011). Originally, granulosa cells from Booroola and Inverdale animals were thought to have an increased responsiveness to FSH, but it has been shown recently that this is not the case. The original data showing increased responsiveness to FSH (Shackell *et al.* 1993) were most likely due to LH contamination in the partially purified preparation of the FSH utilized at that time (McNatty *et al.* 2009). The earlier onset of LH responsiveness in both Inverdale and Booroola ewes permits smaller follicles to survive during the time of suppressed gonadotrophin secretion that occurs during selection of the ovulatory follicle. In this manner, more follicles can advance to ovulation.

Studies have also been undertaken to determine how the *BMPR1B* receptor mutation affects the responsiveness of granulosa cells to BMPs. While there are some differences in the literature regarding which BMPs are produced by the ovine ovarian follicle, it is clear that there are other potential ligands in addition to *BMP15* for *BMPR1B* in growing ovarian follicles (Campbell *et al.* 2006, Juengel *et al.* 2006). Another factor that complicates the interpretation of the results comparing responsiveness of granulosa cells from ewes homozygous for the Booroola mutation to their wild-type contemporaries is the aforementioned differences in the diameter of the ovulatory follicle. Therefore, follicles of similar diameter are likely to represent different developmental stages when comparing Booroola to wild-type animals. Notwithstanding these complexities, granulosa and theca cells in small antral follicles from ewes homozygous for the Booroola mutation appear to be more sensitive to BMPs as well as IGF and gonadotrophins (Campbell *et al.* 2006). Based on the differential activities of the BMPs, this increased sensitivity was proposed to underlie the enhanced differentiation of ovarian follicles in ewes homozygous for the Booroola mutation. The increased sensitivity to BMPs in homozygous Booroola animals has also been observed in pituitary cells (Young *et al.* 2008), supporting the hypothesis that the mutation increases sensitivity of the receptor to BMPs. However, others showed a decreased sensitivity to GDF5 and BMP4 in granulosa cells from small follicles from homozygous Booroola

ewes when assessing their effects on progesterone production (Fabre *et al.* 2003). Again, interpretation of these data is complicated by differences in developmental stages for the same-sized follicles as responsiveness to GDF5 and BMP4 also decreased as follicular size increased. Thus, it is uncertain whether the mutation in the receptor increases or decreases the sensitivity of the receptor to its ligands.

Both Booroola and Inverdale ewes treated with exogenous gonadotrophins (i.e. treatment to induce multiple ovulations) show an increased ovulation rate response when compared with their wild-type contemporaries (McNatty *et al.* 2006a). In preliminary studies, increased ovulation rates following administration of exogenous gonadotrophins have also been observed in ewes heterozygous for the Woodlands gene when compared with wild-type contemporaries (J L Juengel, unpublished results), suggesting that the increased responsiveness to exogenous gonadotrophins *in vivo* is a common feature of animals with mutations that affect the TGFB superfamily pathway. It was recently discovered that there may be a second gene affecting ovulation rate in the New Zealand flock of Booroola ewes. Animals thought to be carrying the second putative gene that are also heterozygous for the Booroola mutation have an enhanced basal ovulation rate but lack the increased responsiveness to exogenous gonadotrophins observed in ewes carrying only the Booroola mutation (Juengel *et al.* 2008). Thus, the increased responsiveness *in vivo* to exogenous gonadotrophins observed in the ewes with mutations affecting the TGFB superfamily pathway is unlikely to be simply related to the increased basal ovulation rates of these animals. The physiological mechanisms of action that underlie the increased responsiveness to exogenous gonadotrophins are unclear. The increased ovulation rate observed in ewes with mutations in TGFB superfamily pathway may relate to the ability of FSH to recruit more follicles for preovulatory maturation from the relatively large pool of small antral follicles (Dufour *et al.* 2000). However, it is important to note that the effects of the Booroola mutation on follicle numbers are inconsistent (Smith *et al.* 1994, Ruoss *et al.* 2009). In addition, the acquisition of LH responsiveness in granulosa cells in a greater proportion of the pool of small antral follicles may allow more follicles to undergo preovulatory maturation (McNatty *et al.* 2009, Crawford *et al.* 2011).

Embryo transfer studies have shown that the increased ovulation rate in response to exogenous gonadotrophins resulted in an increase in transferable embryos in the Booroola, but not in the Inverdale, ewes (McNatty *et al.* 2006a). The inability of the increased ovulation rate observed in the Inverdale animals to translate into a significant increase in transferable embryos was an interesting observation. While no differences in embryo survival have been noted in animals heterozygous for inactivating mutations in *BMP15* (Davis *et al.* 1993), or

those immunized against BMP15 (Juengel *et al.* 2011b), addition of BMP15 to culture media improves blastocyst rates in IVM/IVF procedures (Hussein *et al.* 2006). Thus, it may be that reduced BMP15 secretion by the oocyte results in poorer oocyte quality when the system is 'stressed', as might be expected when exogenous gonadotrophin is used to recruit a greater number of follicles for ovulation. This effect could be independent of the regulation of the number of follicles ovulated. The results from the Booroola ewes are more difficult to interpret, as these embryo transfer studies were carried out prior to identification of the mutation and may have been confounded by the method used to determine the phenotype of the ewes. Genotype selection was based on an extreme ovulation rate difference relative to wild types (e.g. ewes classified as homozygous when ovulation rate ≥ 7 and wild type when ≤ 2). The recent findings of a potential second gene increasing ovulation rate in the flock makes it likely that at least some of these animals may have been carrying a second gene influencing selection of the ovulatory follicle.

The use of immunization to mimic the phenotypes observed in ewes with mutations in GDF9, BMP15 and BMBR1B

The phenotypes of ewes with mutations affecting the TGFB superfamily have potential benefits for modulating fertility in a diverse range of mammals. Consequently, experimental procedures to mimic the infertile and highly fecund phenotypes have been explored in animals not carrying these mutations. As it was known that mutations in *BMP15* were inactivating, immunoneutralization of the protein was a logical strategy to emulate the effects of the mutation. Another strategic approach was to inhibit binding of BMP15 to target cells using antibodies specific for the extracellular domain of its receptor BMPR1B. While initial studies using active immunization against BMP15 and GDF9 were promising (Juengel *et al.* 2002), blocking the binding of these proteins to the BMPR1B receptor using antibodies directed against the extracellular domain did not show consistent effects on ovulation rate (J L Juengel & K P McNatty, unpublished results). Thus, subsequent studies were focused on immunization strategies against GDF9 and BMP15.

Three possible outcomes were examined with the immunization strategies: i) cessation of reproductive cycles; ii) increased ovulation rate during a natural cycle and iii) increased responsiveness to a superovulation regimen. Considerable progress has been made in using active immunization to stop reproductive cycles and increase ovulation rate; however, the third option remains elusive. Peptide mapping identified the N-terminal of the mature polypeptides as the most efficacious in generating high-titre antibodies capable of

specific neutralization of either GDF9 or BMP15 (Juengel *et al.* 2002, McNatty *et al.* 2007). Furthermore, through the use of different adjuvants (oil based or water based), the amount of GDF9 or BMP15 able to be neutralized could be modulated to either cause cessation of reproductive cycles (Juengel *et al.* 2002, McNatty *et al.* 2007) or increased ovulation rate (Juengel *et al.* 2004a). Immunization using a water-based adjuvant did not negatively affect embryo survival, although the animal numbers in that study were not large enough to observe subtle differences (Juengel *et al.* 2004a). Initial studies indicated that the increased ovulation rate observed in BMP15-immunized animals could result in an increased lambing rate (Juengel *et al.* 2004a). However, preliminary analysis from ewes immunized annually over a 3-year trial period has shown that, while increased ovulation rates were observed, the lambing rates in BMP15-immunized ewes became increasingly unfavourable over time (increased non-pregnant ewes and ewes with three or more lambs) when compared with the commercially available twinning vaccines utilizing an androstenedione-based antigen (Juengel *et al.* 2011b).

All attempts to increase the superovulation rate response to exogenous FSH through immunization against either BMP15 or GDF9 have proven unsuccessful (J L Juengel & K P McNatty, unpublished results, Juengel *et al.* 2011a). For BMP15-immunized ewes, the ovarian follicular populations have been studied to determine why immunized ewes do not show an increased responsiveness to exogenous gonadotrophins (Juengel *et al.* 2011a). While the follicles from BMP15 ewes were smaller in diameter with fewer granulosa cells than control ewes, their responsiveness to gonadotrophins did not mimic what was observed in the Inverdale animal. Although the follicles tended to be more responsive to human chorionic gonadotrophin (hCG, used to measure LH responsiveness), the basal cAMP production was also elevated. Thus, the follicles may have been prematurely luteinizing, thereby preventing increased responsiveness to exogenous gonadotrophins. However, this possibility could not have interfered with the increased ovulation rate in natural (i.e. not stimulated with gonadotrophins) cycles (Juengel *et al.* 2004a). While attempts to improve the efficacy of current superovulation regimens have not been successful, individual animals undergoing natural reproductive cycles were noted with very high ovulation rates (>10). The current working hypothesis is that ovulation rate increases in response to decreasing amounts of biologically active BMP15 or GDF9 until a critical low threshold is reached, at which follicular growth ceases (McNatty *et al.* 2004). Thus, immunization strategies to reduce the amount of BMP15 and/or GDF9 to levels just above the critical threshold could form the basis of new superovulation protocols not involving exogenous FSH.

Immunization against BMP15 and GDF9 has also been examined in cattle. Preliminary studies showed

responses that were similar, although not identical, to sheep when examining the effects during the oestrous cycle, with some animals progressing towards anovulation whereas others experienced high ovulation rates (Juengel *et al.* 2007, 2009). The results in cattle indicated quite clearly that GDF9 and BMP15 proteins are involved in the regulation of follicular growth and/or ovulation rate. However, there were practical difficulties in controlling these effects. These were due to the variability of responses experienced as well as the difficulty in inducing an appropriately high humoral immune response (Juengel *et al.* 2009).

Effects of mutations in genes controlling ovulation rate on embryo/fetal survival, lamb birth weight, lamb survival and lamb growth

The relationships between ovulation rate and embryo/fetal survival, lamb birth weight, lamb survival and lamb growth are complex. As ovulation rate increases, the individual chance of any one ova developing into a lamb decreases (Hanrahan 1982). Likewise, increased litter size is related to decreased birth weights, and small birth weights are linked to higher lamb mortality and lower lamb growth rates (Morris *et al.* 2003, Kerslake *et al.* 2005, Peterson *et al.* 2006). When these parameters were examined in ewes with altered BMP15 bioactivity, the aforementioned relationships appeared similar. In Inverdale ewes (Davis *et al.* 1993, Demmers *et al.* 2011), embryo/fetus survival or lamb performance was not altered and immunization against BMP15 also did not appear to alter these factors either if changes in ovulation rate/litter size were accounted for (Juengel *et al.* 2004a, 2011b). Little data are available regarding the effects of mutations in the *GDF9* gene on these factors (Juengel *et al.* 2004a, Nicol *et al.* 2009), but given that GDF9 and BMP15 seem to act cooperatively in sheep, it seems likely that mutations in *GDF9* would also not affect these relationships.

The picture is more complex in Booroola animals. Given that *BMPR1B* is expressed throughout the body (Wilson *et al.* 2001), in contrast to the limited expression of BMP15 and GDF9 in the oocyte, some effect of the mutation in *BMPR1B* on lamb performance independent of ovulation rate changes could be postulated. In one study, while birth weight and growth were similar between non-carriers and heterozygous carriers of the *BMPR1B* mutation, homozygous carriers were smaller at birth, had reduced post-weaning growth rates and lower mature body weights (Gootwine *et al.* 2006). Other studies have shown that birth weight, growth and 12-month body weight were affected in both the heterozygous and the homozygous carriers (Kumar *et al.* 2008). However, Willingham *et al.* (2002) did not find an effect on birth weight or growth rate in Booroola wethers that was independent of litter size at birth/

rearing. Similarly, Kleemann *et al.* (1985) concluded that the Booroola gene did not appear to affect growth or carcass characteristics. Genotype did not appear to affect lamb survival (Gootwine *et al.* 2008). Taken together, it seems most likely that changes in ovulation rate, and thus litter size at birth and during rearing, are the major influences on lamb performance in Booroola ewes with the potential for more subtle effects on these factors occurring independently of changes in litter size.

The relevance of sheep lines with mutations in single genes to understanding ovarian function

As stated earlier, both GDF9 and BMP15 have been shown to be essential for ovarian follicular development in sheep, whereas in mice, GDF9, but not BMP15, is essential. This suggests that the actions of these proteins may differ between species. Naturally occurring mutations of both *BMP15* and *GDF9* have been identified in humans and those in *BMP15* that result in a loss of biologically active protein lead to premature ovarian failure (Di Pasquale *et al.* 2004, 2006, Dixit *et al.* 2006). Mutations in *GDF9* were also linked with sterility (Dixit *et al.* 2005, Laissue *et al.* 2006) and dizygotic twinning (Palmer *et al.* 2006) similar to what has been observed in sheep. While variation in *BMP15* was not found to be a major underlying cause of dizygotic twinning in humans, three rare mutations were observed in mothers of spontaneous dizygotic twins (MODZT) but not in controls. However, association between these mutations and twinning could not be established (Zhao *et al.* 2008). Similarly, while mutations in *BMPR1B* were observed in MODZT, they were only marginally associated with increased twinning (Luong *et al.* 2011). Similar to the increased responsiveness to gonadotrophins observed in sheep heterozygous for a *BMP15* mutation or homozygous for the *BMPR1B* mutation (McNatty *et al.* 2006a), mutations in the human population in *BMP15* have also been linked weakly to a higher response to FSH (Moron *et al.* 2006, Hanevik *et al.* 2011). In addition, the expression patterns for *BMP15* and *GDF9*, as well as their receptors, are similar between sheep and humans (Juengel & McNatty 2005). Thus, in general terms, the actions of GDF9 and BMP15 appear similar between sheep and humans providing evidence that understanding the role of these proteins in the sheep model is not only important for regulating reproductive efficiency in sheep but also for providing insights into control of ovarian follicular development in humans.

Studies examining the functions of BMP15 and GDF9 *in vitro* also support the relevance of the sheep models in understanding control of ovarian follicular development in multiple species. Generally, GDF9 and BMP15 (alone or combined) stimulate granulosa cell proliferation, inhibit progesterone production and stimulate inhibin production in both sheep and human granulosa cells

(Juengel & McNatty 2005). While GDF9 and BMP15 have some activity on their own, both human and ovine GDF9 and BMP15 together have been shown to work synergistically (McNatty *et al.* 2005c, Peng *et al.* 2013). Taken together with the similarity in expression patterns of GDF9 and BMP15 in the oocytes of growing follicles in both humans and sheep, it is most likely that in sheep and humans, GDF9 and BMP15 work as a functional unit (Juengel & McNatty 2005, Peng *et al.* 2013).

Other factors regulating cellular response to ligands are expression of the receptors for the ligands and which cellular signalling pathways are activated on ligand binding. Members of the TGF β superfamily typically interact with a receptor complex composed of two types (type 1 and 2) of membrane-bound serine–threonine kinases (de Caestecker 2004, ten Dijke & Hill 2004). Both GDF9 and BMP15 use BMPR2 for the type 2 receptor (Vitt *et al.* 2002, Moore *et al.* 2003, Edwards *et al.* 2008). BMP15 signals through BMPR1B as its type 1 receptor (Moore *et al.* 2003, Pulkki *et al.* 2012) with GDF9 signalling through the TGFBR1 receptor (Mazebourg *et al.* 2004). ACVR1B has been proposed to be possibly involved in signalling when both BMP15 and GDF9 are present (Peng *et al.* 2013). The classical signalling pathway used by these receptors is the SMAD signalling pathway with two receptor-associated subfamilies (Miyazawa *et al.* 2002, ten Dijke & Hill 2004). Interestingly, BMPR1B activates the classical BMP subfamily pathway (Smad 1/5/8) whereas TGFBR1 activates a classical activin/TGF β subfamily pathway (Smad 2/3). Thus, the pairing of BMPR2 with TGFBR1 for GDF9 signalling is an unusual pairing. Other non-classical pathways such as MAP kinases and NF- κ B may also be involved in GDF9 and BMP15 signalling (Moore *et al.* 2003, Reader *et al.* 2011).

Recently, activation of cellular signalling pathways in rat granulosa cells following treatment with a GDF9 and BMP15 combination (GDF9+BMP15) was shown to differ depending on whether the GDF9 and BMP15 were derived from ovine or murine sequence (Reader *et al.* 2011). While the classical Smad 2/3 pathways were shown to be critical for granulosa cell proliferation for both mouse and ovine GDF9+BMP15, non-classical signalling pathways were also identified with NF- κ B being critical for activity of ovine, but not mouse, protein. Similarly, inhibition of the ERK–MAPK pathway partially blocks activity of mouse, but not ovine, GDF9+BMP15. Activation of the Smad 2/3 pathway was also shown to be involved in the regulation of expression of genes involved in cumulus cell expansion by human GDF9+BMP15 (Peng *et al.* 2013). No evidence for the role of NF- κ B was observed when studying the combined actions of mouse GDF9 and human BMP15 (Mottershead *et al.* 2012); however, signalling through NF- κ B was linked to the species of origin of GDF9 (Reader *et al.* 2011). Preliminary studies carried out in our laboratory also identified Smad 2/3 as a classical signalling pathway and

NF- κ B and JNK as non-classical signalling pathways potentially involved in stimulation of rat granulosa cell proliferation by human GDF9+BMP15, indicating similarity between the human and ovine proteins.

There is still considerable knowledge to be gained regarding the regulation and function of BMP15 and GDF9. These include, but are not limited to, a better understanding of the biologically active forms of BMP15 and GDF9 produced by the oocyte *in vivo*. Analysis of GDF9 and BMP15, in follicular fluid, oocyte lysates and from cell lines expressing GDF9 or BMP15, indicates that GDF9 and BMP15 may be secreted as a complex mix of forms (Liao *et al.* 2003, 2004, McNatty *et al.* 2006b, Edwards *et al.* 2008, McIntosh *et al.* 2008, Lin *et al.* 2012). New data are emerging regarding the effects of post-translational modifications on the activity of GDF9 and BMP15 (McMahon *et al.* 2008) and the role of sequence in the proregion in regulating activity of the mature region. These may operate via control of processing of the protein and regulating latency of the mature protein or potentially direct actions (McIntosh *et al.* 2008, 2012, Tibaldi *et al.* 2010, Simpson *et al.* 2012, Al-Musawi *et al.* 2013). The relative importance of the effects of GDF9 and BMP15 on mural vs cumulus granulosa cell function is also unclear, and sometimes debated. Being produced by the oocyte, the cumulus cells are clearly exposed to high concentrations of these growth factors and their role in regulating cumulus cell function and oocyte quality has been shown (Gilchrist *et al.* 2008, Gilchrist 2011). However, the presence of these factors in follicular fluid and the ability of passive immunization against BMP15 to affect ovulation rate demonstrate an essential role at the level of the mural granulosa cells also (Juengel *et al.* 2002). Understanding these and other factors better will be essential to fully understand the role of GDF9 and BMP15 in regulating follicular development.

Thus, while there is still much to learn regarding the actions of BMP15 and GDF9, the sheep models have proven invaluable in understanding how the oocyte-derived growth factors regulate ovarian follicular development, ovulation rate and oocyte function. While the knock-out *Gdf9* mouse (Dong *et al.* 1996) clearly set us on the track of understanding the essential role of these growth factors in regulating female fertility, it could be argued that the less well-known Hanna sheep line, which is in essence a naturally occurring sheep knock-out of *BMP15*, together with the Inverdale line, provided the first unequivocal evidence for the essential role of BMP15 in regulating female fertility (Galloway *et al.* 2000). The discovery that GDF9 was also essential for ovarian follicular development and regulating ovulation rate in sheep (Juengel *et al.* 2002, Hanrahan *et al.* 2004) shortly thereafter set us on the track to determine how the two oocyte-derived growth factors work together to control fertility.

Declaration of interest

AgResearch holds patents related to the use of BMP15 and GDF9 in regulating ovarian function. J L Juengel, G H Davis and K P McNatty are listed as inventors on these patents; however, all have assigned their ownership to AgResearch Ltd.

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