BIOLOGY OF REPRODUCTION **70,** 900–909 (2004)
Published online before print 19 November 2003.
DOI 10.1095/biolreprod.103.023093

Mutations in the Genes for Oocyte-Derived Growth Factors GDF9 and BMP15 Are Associated with Both Increased Ovulation Rate and Sterility in Cambridge and Belclare Sheep (*Ovis aries*)[1]

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ABSTRACT

Belclare and Cambridge are prolific sheep breeds, the origins of which involved selecting ewes with exceptionally high litter size records from commercial flocks. The variation in ovulation rate in both breeds is consistent with segregation of a gene (or genes) with a large effect on this trait. Sterile ewes, due to a failure of normal ovarian follicle development, occur in both breeds. New naturally occurring mutations in genes for the oocyte-derived growth factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are described. These mutations are associated with increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in both breeds. This is the first time that a mutation in the gene for GDF9 has been found that causes increased ovulation rate and infertility in a manner similar to inactivating mutations in BMP15, and shows that GDF9 is essential for normal folliculogenesis in sheep. Furthermore, it is shown, for the first time in any species, that individuals with mutations in both *GDF9* and *BMP15* have a greater ovulation rate than sheep with either of the mutations separately.

follicle, follicular development, growth factors, ovary, ovulation

INTRODUCTION

Genetic variation in ovulation rate in sheep has been widely documented and the evidence shows substantial differences among breeds and in a number of cases exceptional variation within breeds/strains [1]. The latter phenomenon can be explained by segregation of a gene with a large effect on ovarian function. This hypothesis provided an explanation for high prolificacy of Booroola sheep [2, 3]. Subsequently, putative major genes were invoked to explain the increased litter size and/or ovulation rate in a variety of breeds/strains, including Inverdale [4], Cambridge [5], Thoka [6], Javanese [7], Olkuska [8], Belclare [9], Lacaune [10], and Woodlands [11] sheep. The major gene involved in the Inverdale was shown to be X-linked [4] and it was also shown that homozygous carrier ewes were sterile due to ovarian hypoplasia reflecting a failure of ovarian follicles to progress beyond the primary stage of follicle development [12, 13]. In contrast, the Booroola gene is on chromosome 6 [14] and has an essentially additive effect on ovulation rate [15]. The genes responsible for the Booroola and Inverdale effects were recently identified. The Booroola gene (*FecB*) is a mutation in the receptor for a bone morphogenetic protein (BMPR1B) [16–18]. The Inverdale effect is due to mutations in an oocyte-derived growth factor gene *BMP15* (also known as growth differentiation factor 9B [*GDF9B*]) [19]. Two different independent point mutations in *BMP15* have been identified (called Inverdale [$FecX^I$] and Hanna [$FecX^H$]) [20]. These discoveries enabled testing of other populations carrying putative major genes (Thoka, Belclare, Cambridge, Javanese, Olkuska, Woodlands, and Lacaune) for the presence of the Booroola and Inverdale/Hanna mutations. The Javanese sheep were the only case where one of these mutations (Booroola) was detected [21].

Cambridge and Belclare sheep are characterized by a high ovulation rate with extreme variation among individuals, consistent with segregating major genes, and it has been hypothesized that two major genes could be involved in both breeds [9, 22]. In addition, female sterility due to ovarian hypoplasia, similar to that in Inverdale sheep [12, 13], has been observed in both breeds [9, 23]. Studies of the inheritance patterns of the ovarian hypoplasia have shown that an autosomal gene was likely to be involved in both populations [24]. Furthermore, histological analysis of hypoplastic ovaries from both populations showed some significant differences from the abnormalities associated with the Inverdale mutation [12, 13], such as the presence of follicles with an antrum and abnormal oocyte surrounded by abnormally dispersed layers of cells and oocytes with thickened zonae pellucidae (unpublished data).

Two likely candidates for the phenotypes are the genes growth differentiation factor 9 (*GDF9*) and *BMP15*. Both genes are members of the transforming growth factor beta (TGFβ) superfamily, coding for distinct proteins, the expression of which in ovarian tissue is exclusively in the oocyte of the developing follicle, and which play an essential role in mammalian fertility. GDF9 is expressed in oocytes from the primary stage of follicular development until

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Received: 18 September 2003.
First decision: 20 October 2003.
Accepted: 12 November 2003.

ISSN: 0006-3363. http://www.biolreprod.org

ovulation [25, 26]. Female GDF9 knockout mice (*GDF9*$^{-/-}$) are infertile due to a block in follicular development at the primary stage [27]. BMP15 is expressed in mouse oocytes at the same time as GDF9, but in human primary follicles slightly later than GDF9 [26, 28]. BMP15 does not appear to be crucial for mouse folliculogenesis as knockout female mice (*BMP15*$^{-/-}$) are fertile [29], although fecundity is somewhat reduced. However, BMP15 is essential for folliculogenesis in sheep [19]. In sheep, it is also clear that heterozygotes carrying inactivating mutations in only one copy of BMP15 have an increased ovulation rate [19].

Sheep *GDF9* has been mapped to sheep chromosome 5 [30]. The gene spans about 2.5 kilobases (kb) and contains 2 exons separated by a single 1126-base pair (bp) intron and encodes a prepropeptide of 453 amino acid residues. The active mature peptide is 135 amino acids long [31]. Sheep *BMP15* maps to the X chromosome [19]. The full length coding sequence of 1179 nucleotides is contained in two exons, separated by an intron of about 5.4 kb, and encodes a prepropeptide of 393 amino acid residues. The active mature peptide is 125 amino acids long [19].

The present study used a candidate gene approach to identify the basis for the ovarian hypoplasia in Cambridge and Belclare sheep. The primary candidate gene was *GDF9*, based on the role of this gene in follicle development in mice [27] and the prior evidence that an autosomal gene was involved. We also decided to examine *BMP15* for mutations other than those involved in the Inverdale/Hanna sheep.

MATERIALS AND METHODS

Animals

The origins of the Belclare and Cambridge populations maintained at the Research Centre, Athenry, Ireland, and which provided all of the animals used in this study, have been described by Hanrahan [9]. Briefly, both breeds owe their origin to selection of foundation ewes with exceptionally high litter size records from commercial flocks. These foundation animals were used to establish closed flocks that were selected for high litter size—the High Fertility line in Ireland [32] and the Cambridge breed in Britain [33]. The High Fertility flock was subsequently combined with an interbred Finn × Galway line and selected Lleyn sheep (selected for high litter size) to generate a composite breed called Belclare. Subsequent measurements of ovulation rate in this composite breed revealed the presence of ewes with exceptionally high ovulation rate and these were used to establish a subline (F700) for the investigation of the role of a putative major gene [9]. This subline provided the Belclare animals used in the present study. The Cambridge flock at the Sheep Research Centre was established by selecting ewes with the highest ovulation rates and male progeny of high-ovulation-rate ewes from the parent flock of the Cambridge breed that was located in the University of Wales at Bangor [9]. The F700-Belclare and Cambridge flocks at the Sheep Research Centre are self-contained with 4–6 males used each year; each sire is generally replaced by a son to minimize inbreeding. The study encompassed ewes born up to and including 1999.

To generate evidence on the involvement of genes with large effects on ovulation rate, rams from the Belclare were progeny tested for ovulation rate by crossing with ewes from two nonprolific breeds (Galway and Scottish Blackface). A total of 32 Belclare rams were involved and the test-cross daughters were born over the period 1990–1997, but DNA was only available from 10 of these rams.

All the ewes produced for the progeny test evaluation were assembled into an evaluation flock as ewe lambs and were retained in that flock until they reached 4.5 yr of age. Ovulation rate was assessed each breeding season from the ewe lamb stage, but first joining with rams was at 1.5 yr of age.

Ovarian examinations to determine ovulation rate were carried out by midventral laparoscopy under license issued in accordance with Irish and European Union legislation (Cruelty to Animals Act, 1876, and European Community Directive, 86/609/EC), and all animals were managed in accordance with the guidelines for the accommodation and care of animals under Article 5 of the Directive.

Blood samples were retained for DNA extraction from the sterile Cambridge ewes born in 1990 and later years and from essentially all of the F700-Belclare sterile females born since 1993. In addition, blood samples for DNA extraction were collected from fertile ewes in these flocks from 1992 onward.

DNA Extraction

Genomic DNA was isolated either from frozen stored buffy coat or directly from white blood cells in whole blood [34]. Parentage of key pedigrees was verified with autosomal sheep microsatellite markers OarHH64 (sheep chromosome 4), OarCP34 (sheep chromosome 3), and OarFCB304 (sheep chromosome 19) [35].

Gene Identification

The primary approach used was to determine the DNA sequence for the candidate genes in DNA from sterile ewes and from their parents and female siblings. In addition, SSCP (single-stranded conformational polymorphism) analysis was carried out on DNA from nine of the Belclare rams involved in the progeny testing program and on test-cross daughters of three of these rams (29, 17, and 12 progeny). A further seven purebred daughters of two of the Belclare rams were also tested along with four of their five dams. Similarly, DNA from two Cambridge rams (one of which had been progeny tested) was also examined by SSCP.

Sequencing and Mutation Detection

The sheep *GDF9* and *BMP15* genes were amplified using the polymerase chain reaction (PCR) with primers designed from published sheep sequences (sheep genomic BMP15 exon 1, AF236078; sheep genomic BMP15 exon 2, AF236079; sheep genomic GDF9 exon 1 and 2, AF078545).

The PCR primers used were as follows: *BMP15* exon 1 (B-13: 5′-CATGCTGCCTTGTCCCAC and B-28: 5′-AGGCAATGTGAAGCCTGACA); *BMP15* exon 2 (B-25: 5′-CAGTTTGTACTGAGCAGGTC, and B-4: 5′-TTCTTGGGAAACCTGAGCTAGC); *GDF9* exon 1 (G-1: 5′-GAATTGAACCTAGCCCACCCAC, and G-4: 5′-AGCCTACATCAACCCATGAGGC); *GDF9* exon 2 (G-5: 5′-ATCCCACCCTGACGTTTAAGGC, and G-7: 5′-TCCTCCCAAAGGCATAGACAGG). The resulting PCR products were sequenced on an ABI 373 sequencer (Applied Biosystems, Foster City, CA).

Single-Stranded Conformational Polymorphism Detection

BMP15 genotypes were determined by analysis of three nucleotide fragments that spanned most of exon 2. Fragments analyzed by SSCP were Exon 2:353bp (B15-359: 5′-CGCTTTGCTCTTGTTCCCTCT, and B15-691: 5′-CCTCACTACCTCTTGGCTGCT), Exon 2:273bp (B15-664: 5′-GGGTTCTACGACTCCGCTTC, and B15-916: 5′-GGTTACTTTCAGGCCCATCAT) and Exon 2:312bp (B15-915: 5′-CATGATGGGCCTGAAAGTAAC, and B15-1205: 5′-GGCAATCATACCCTCATACTCC). Primers were designed from nucleotide sequence GenBank Accession number AF236079 and primer names correspond to nucleotide position within that sequence.

GDF9 genotypes were determined by analysis of five nucleotide fragments which spanned exon 1, part of the intron, and most of exon 2. Fragments analyzed by SSCP were Exon 1:462bp (G9-1734: 5′-GAAGACTGGTATGGGGAAATG, and G9-2175: 5′-CCAATCTGCTCCTACACACCT), Intron:294bp (G9-2676: 5′-GTGTGAGAGAGATGGGAGCA, and G9-2947: 5′-AAGAGGAAAACTATCAAAAGACA), Exon 2: 296bp (G9-3270: 5′-TGGCATTACTGTTGGATTGTTTT, and G9-3546: 5′-CAAGAGGAGCCGTCACATCA), Exon 2:206bp (G9-3543: 5′-GATTGATGTGACGGCTCCTCT, and G9-3728: 5′-GGGAATGCCACC TGTGAAAAG), Exon 2:221bp (G9-3939: 5′-TCTTTTTCCCCA GAATGAATGT, and G9-4140: 5′-CACAGGATGGTCTTGGCACT). Primers were designed from nucleotide sequence GenBank Accession number AF078545 and primer names correspond to nucleotide position within that sequence.

Amplification was for 30 cycles in a 40-μl reaction mixture, with 150 ng of genomic DNA and 1.5 mM or 3 mM magnesium at an annealing temperature of 55–58°C. PCR fragments were analyzed by SSCP in polyacrylamide gels with overnight migration at 9–15 V/cm, 15°C.

Nomenclature of Variants and Mutated Alleles

For purposes of clarity, we have labeled the DNA variants G1–G8 in *GDF9* and B1–B4 in *BMP15* (see Table 1). We propose the following

TABLE 1. Polymorphic sequence variations in *GDF9* (growth differentiation factor 9) and *BMP15* (bone morphogenetic protein 15) within the Cambridge and F700-Belclare flocks.

Gene	Variant[a]	Base change	Coding base (bp)	Coding residue (aa)	Mature peptide residue (aa)	Amino acid change
GDF9	G1	G-A	260	87		Arg (R)–His (H)
	G2	C-T	471	157		Unchanged Val (V)
	G3	G-A	477	159		Unchanged Leu (L)
	G4	G-A	721	241		Glu (E)–Lys (K)
	G5	A-G	978	326	8	Unchanged Glu (E)
	G6	G-A	994	332	14	Val (V)–Ile (I)
	G7	G-A	1111	371	53	Val (V)–Met (M)
	G8	**C-T**	**1184**	**395**	**77**	**Ser (S)–Phe (F)**
BMP15	B1	CTT del	28–30	10		Leu deletion
	B2	**C-T**	**718**	**239**		**Gln (Q)–STOP**
	B3	T-C	747	249		Unchanged Pro (P)
	B4	**G-T**	**1100**	**367**	**99**	**Ser (S)–Ile (I)**

[a] The variants in bold are associated with sterility.

nomenclature for the three putative functional mutated alleles based on the current names for existing fecundity genes in sheep [36]. The two *BMP15* alleles are $FecX^G$ for B2, and $FecX^B$ for B4 (*X*, X-chromosome; ^{G}, Galway; ^{B}, Belclare). This is in line with the $FecX^I$ and $FecX^H$ names for Inverdale and Hanna alleles of *BMP15* (*FecX*) [19, 36]. The *GDF9* mutation (G8) we have called $FecG^H$ (*G*, GDF9; ^{H}, high fertility). The wild-type alleles are referred to as $FecX^+$ and $FecG^+$.

Single Nucleotide Polymorphism Detection Assays

The G to A nucleotide change in *GDF9* exon 1 (G1 in Table 1) disrupts a *Hha* I restriction enzyme cleavage site (GCGC to GCAC) at nucleotide 260 of the 462-bp PCR fragment produced by primers G9-1734 and G9-2175. Digestion of 9 μl of PCR product with 3 U *Hha*I in 15-μl final volume was for 6 h at 37°C. Restriction digestion of the PCR product from wild-type animals with *Hha*I resulted in cleavage of the 462-bp product (at two internal *Hha*I sites) into fragments of 52, 156, and 254 bp. However, DNA fragments containing the A nucleotide yield only two fragments (52 and 410 bp). Animals heterozygous for the mutation have fragments of all four sizes (52, 156, 254, and 410 bp).

The remaining single nucleotide polymorphisms (SNPs) in *GDF9* and *BMP15* identified by sequencing did not affect common restriction endonuclease cleavage sites. To screen these polymorphisms through the F700-Belclare and Cambridge flocks, PCR was carried out using primers with single mismatches to generate products containing restriction enzyme sites. The mismatch created in the appropriate primer to create the restriction enzyme cleavage site is underlined in the primer sequences below. In all five assays, amplification was 94°C for 5 min; 35 cycles of 94°C for 30 sec; an annealing step for 40 sec (at the specific temperature stated below for each assay); extension at 72°C for 30 sec; followed by a final extension of 72°C for 4 min. Magnesium concentration was 1.5 mM. Primer sequences and PCR conditions for each assay are below and restriction enzyme digests were carried out as per the manufacturer's instructions.

The *GDF9* G4 variant (G to A nucleotide change) was detected using G4-Sfu1F 5′-GGAATATTCACATGTCTGTAAATTTTACATGTTCG and G4-Sfu3R 5′-GAGGGAATGCCACCTGTGAAAAGCC to amplify a 161-bp PCR product at 63°C. The mutated strand was cleaved with *Sfu* I. The *GDF9* G7 variant (G to A nucleotide change) was detected using G7-Tru1R 5′-CAGTATCGAGGGTTGTATTTGTGTGGGGCCT and G7-Tru3F 5′-GCCTCTGGTTCCAGCTTCAGTC to amplify a 158-bp PCR product at 63°C. The mutated strand was cleaved with *Mse*I. The *GDF9* G8 variant ($FecG^H$) (C to T nucleotide change) was detected using G8-Dde1R: 5′-ATGGATGATGTTCTGCACCATGGTGTGAACCTGA and G8-Dde3F: 5′-CTTTAGTCAGCTGAAGTGGGACAAC to amplify a 139-bp PCR product at 62°C. The wild-type strand was cleaved with *Dde*I. The *BMP15* B2 variant ($FecX^G$) (C to T nucleotide change) was detected using B2-Hinf1F: 5′-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC and B-26: 5′-GATGCAATACTGCCTGCTTG to amplify a 141-bp PCR product at 63°C. The wild-type strand was cleaved with *Hinf* I. The *BMP15* B4 variant ($FecX^B$) (G to T nucleotide change) was detected using B4-Dde1F: 5′-GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA and B-4: 5′-TTCTTGGGAAACCTGAGCTAGC to amplify a 153-bp PCR product at 64°C. The wild-type strand was cleaved with *Dde*I.

Restriction digestion of PCR products resulted in cleavage of the longer primer to produce a 30–35-bp smaller product than the uncleaved fragment. Animals heterozygous for any of the mutations have fragments of both sizes. The digested fragments were separated on a 4% agarose gel and visualized with ethidium bromide staining. The gels were scored for the presence or absence of the mutations. Homozygous, heterozygous, and negative controls were included with each assay.

Statistical Analyses

Analysis of ovulation rate data involved least squares procedures (procedure GLM of SAS 1995; SAS Institute, Cary, NC) to fit models with effects for ewe age, year of record, breed of dam (progeny test data), sire (progeny test data), and genotype of the individual (purebred data) and ewe identity within genotype (purebred data). The term for genotype was a three-digit sequence where each position represented the number of copies (0 or 1) of one of the individual mutations described below. The variation among ewes within genotype was used to calculate standard errors. In the case of progeny test data, the gene effects were estimated by appropriate linear contrasts among the sire means.

RESULTS

Detecting Mutations in Cambridge and F700-Belclare Sheep

To determine whether mutations in *GDF9* or *BMP15* were contributing to sterility, sequence information was obtained for the entire coding sequence of both genes in a subset of Cambridge (n = 9) and F700-Belclare sheep (n = 10). Animals were chosen for full-length sequencing based on their sterility phenotype or their relationship to sterile animals. In addition, mutation detection was also carried out by SSCP analysis independently of the above sequencing to look for a relationship with ovulation rate in test-cross daughters of Belclare rams.

Mutations in GDF9

The combined results from sequence data and SSCP analysis of *GDF9* revealed eight single nucleotide polymorphisms across the entire coding region (G1–G8; Table 1). SSCP analysis identified five fragments that contained conformational differences. These differences correspond to one SNP in exon 1, one SNP in the intron, and five SNPs in exon 2. The mutations within the coding region corresponded exactly to those identified from the sequence data. Table 1 shows, for each polymorphism identified within the coding region, the nucleotide position in the full-length sequence, the position of the amino acid residue involved, and the position of the residue (if any) within the mature coding sequence.

Three of the eight polymorphisms are nucleotide changes that do not result in an altered amino acid (G2 at nucleotide position 471, G3 at nucleotide 477, and G5 at nucle-

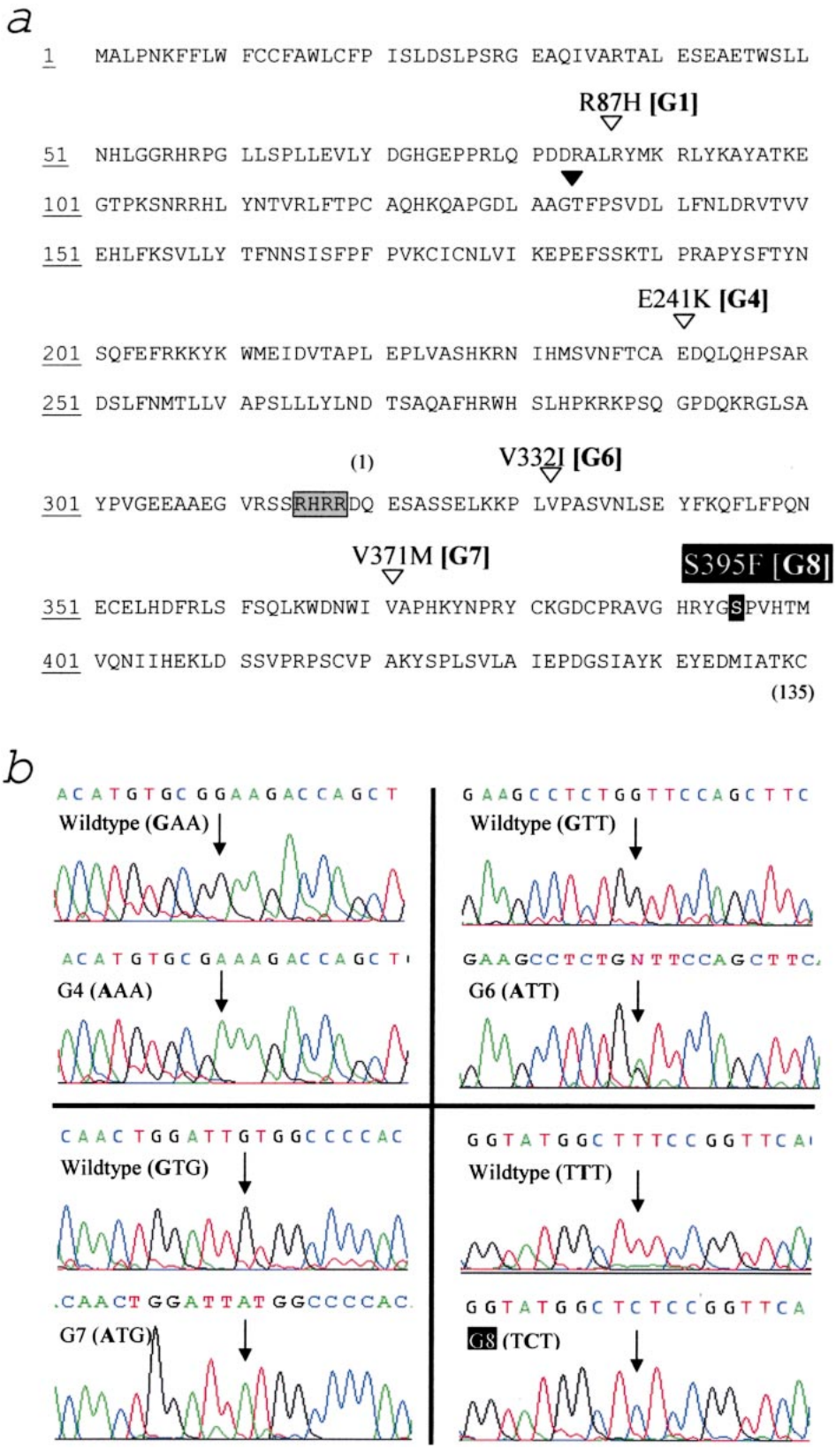


FIG. 1. Irish Cambridge and F700-Belclare sheep GDF9 sequence and mutations. **a**) Predicted amino acid sequence of sheep GDF9 protein. Numbers at the start of each line indicate amino acid positions in the full-length unprocessed protein. Amino acid positions in the mature peptide are in parentheses. The RRHR furin protease cleavage site and predicted start of the mature processed peptide is shaded in gray. The filled triangle indicates the position of the single intron within the gene. The open triangles indicate positions of mutations that confer amino acid substitutions but are not associated with the sterility phenotype. The position of the G8 (*FecG*^{H}) mutation associated with sterility is shaded black. **b**) Nucleotide substitutions of the four *GDF9* mutations that change an amino acid compared with wild-type sheep sequence.

otide position 978). The five remaining nucleotide changes, G1, G4, G6, G7, and G8, give rise to amino acid changes (Table 1 and Fig. 1), although three of them are relatively conservative changes. The G1 arginine to histidine change at amino acid residue 87 in exon 1 substitutes one basic charged polar group with another and occurs at a position before the furin cleavage site for the mature peptide, so is unlikely to affect the activity of the mature protein. Both G6, valine to isoleucine change at amino acid residue 332 of the unprocessed protein (residue 14 of the mature coding region), and G7, valine to methionine at residue 371 of the unprocessed protein (residue 53 of the mature coding re-

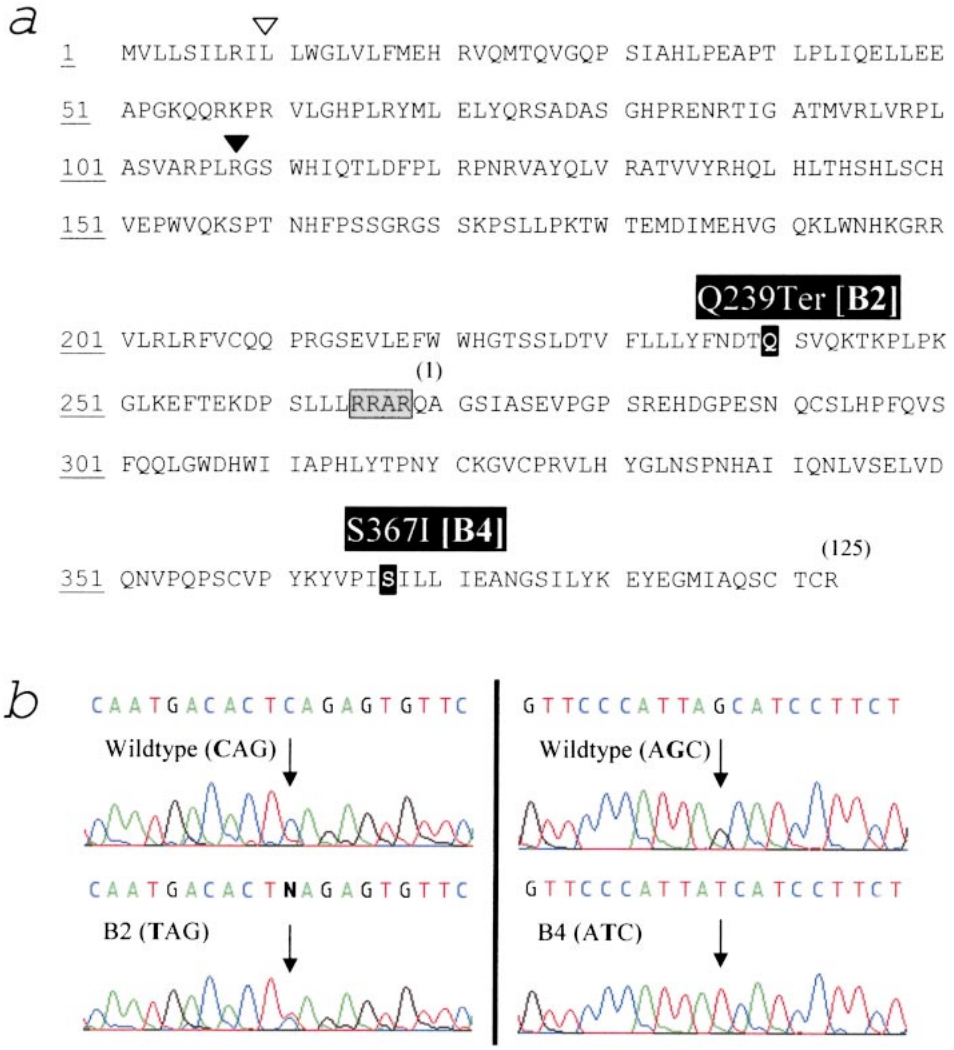


FIG. 2. Irish Cambridge and F700-Belclare sheep BMP15 sequence and mutations. **a**) Predicted amino acid sequence of sheep BMP15 protein. Numbers at the start of each line indicate amino acid positions in the full-length unprocessed protein. Amino acid positions in the mature peptide are in parentheses. The RRAR furin protease cleavage site and predicted start of the mature processed peptide is shaded in gray. The filled triangle indicates the position of the single intron within the gene. The open triangle indicates the position of a single Leu deletion polymorphism. The position of the B2 (*FecX*^{G}) and B4 (*FecX*^{B}) mutations associated with sterility are shaded black. **b**) Nucleotide substitutions of the two *BMP15* mutations that change an amino acid compared with wild-type sheep sequence.

gion), substitute nonpolar groups with nonpolar groups. The remaining two changes result in nonconservative substitutions. G4, glutamic acid to lysine change at amino acid residue 241 of the unprocessed protein, replaces an acidic group with a basic group, but this occurs at a position before the furin processing site and is unlikely to affect the mature active coding region. However, G8, serine to phenylalanine change at residue 395, replaces an uncharged polar group with a nonpolar group at residue 77 of the mature coding region (Fig. 1).

Mutations in BMP15

Both DNA sequencing and independent SSCP analysis of *BMP15* in Cambridge and F700-Belclare sheep revealed four polymorphisms across the entire coding region (B1–B4; Table 1). The first of these (B1) is a previously described leucine deletion polymorphism (without any phenotypic effect) in the predicted signal sequence [19] whereby some sheep have two leucine codons (CTT) at this position and other sheep have only one. One other nucleotide change (B3) does not result in an altered amino acid (nucleotide position 747). The remaining two nucleotide changes (B2 and B4) give rise to more critical changes in the protein (Table 1 and Fig. 2). The C to T change (B2) at nucleotide 718 introduces a premature stop codon in the place of glutamic acid at amino acid residue 239 of the unprocessed protein, which presumably results in complete loss of BMP15 function. The G to T change (B4) at nucleotide 1100 changes the serine residue at amino acid 99 of the mature active protein (residue 367 of the unprocessed protein) to an isoleucine, thereby substituting an uncharged polar group with a nonpolar group (Fig. 2).

Incidence of Mutations in the Flocks

Initial sequencing of a smaller number of animals from each breed identified the G2, G3, G4, G7, and G8 nucleotide changes in *GDF9* and the B2, B3, and B4 changes in *BMP15*. Restriction fragment length polymorphism assays to detect the specific SNPs were developed (as described in *Methods*) for G4, G7, G8 (*GDF9*), B2, and B4 (*BMP15*), and these assays were carried out on larger numbers of animals (Table 2). Subsequent sequencing of full-length *GDF9* and *BMP15* in more animals revealed the G5 and G6 nucleotide changes in *GDF9* in some Cambridge sheep but not in the Belclares. Independent SSCP analysis identified the G1 polymorphism in exon 1 of *GDF9* in one ram, and this was also screened through further animals. G1 was found to be associated with the wild-type alleles in this ram and his test-cross progeny and not associated with ovulation rate. The B1 leucine deletion polymorphism in the prepro region of *BMP15* was only detected in a few animals.

TABLE 2. Genotype analysis of nucleotide changes in *BMP15* and *GDF9* genes from Cambridge and F700-Belclare sheep.[a]

	BMP15			GDF9						
Variant	**B2**	B3	**B4**	G2	G3	G4	G5	G6	G7	**G8**
F700-Belclare	9	2	71	6	6	13	0	0	2	11
	(83)	(13)	(86)	(10)	(10)	(29)	(10)	(10)	(19)	(86)
Cambridge	74	0	0	0	7	1	3	2	7	95
	(129)	(9)	(131)	(9)	(9)	(26)	(9)	(9)	(24)	(126)

[a] Numbers of sheep carrying at least one copy of the variant. Numbers in parentheses indicate total number of sheep genotyped for each variant.

Homozygous Mutations Relate to Sterility

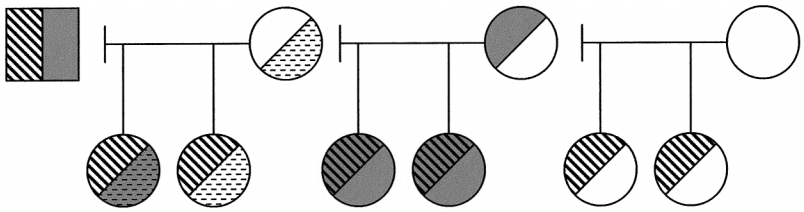
The presence or absence of each of these nucleotide changes was examined in relation to sterility of all the animals tested. This revealed that only the G8 change in *GDF9* and the B2 and B4 changes in *BMP15* were associated with the sterility phenotype. Female sheep that were homozygous for G8 were all sterile; female sheep that were homozygous for B2 or homozygous for B4 were sterile; female sheep that were heterozygous for B2 and B4 simultaneously (i.e., one allele with each mutation, B2/B4) were sterile. Figures 3 and 4 show small F700-Belclare and Cambridge pedigrees, illustrating what was seen in the larger set of animals.

All three functional mutations (B2, B4, and G8) were detected in the F700-Belclare flock, as illustrated in Figure 3. This F700-Belclare pedigree was sired by R830 carrying the *BMP15* B4 mutation on the X chromosome and the *GDF9* G8 mutation on chromosome 5 but not carrying the *BMP15* B2 mutation. Two daughters (930458 and 930459) were sterile due to inactive copies of *BMP15* from both parents (B2/B4). The other two sterile daughters (930811 and 930812) appear to be sterile due to having inherited the *GDF9* G8 mutation from both parents, as their infertility cannot be explained by *BMP15* mutations. Offspring 930810 and 948302 were not homozygous for any of these mutations and were fertile.

The B4 mutation was not seen in any animals tested from the Cambridge flock. Figure 4 illustrates two Cambridge pedigrees. Sire 962101 carried the *BMP15* B2 mutation on the X chromosome and the *GDF9* G8 mutation on chromosome 5 but did not have the *BMP15* B4 mutation. Sire 930142 carried the *BMP15* B2 mutation on his X chromosome and the *GDF9* G8 mutation on each copy of chromosome 5. Sterile daughters are either homozygous for the *BMP15* B2 mutation or the *GDF9* G8 mutation or both (daughters 997634 and 997635).

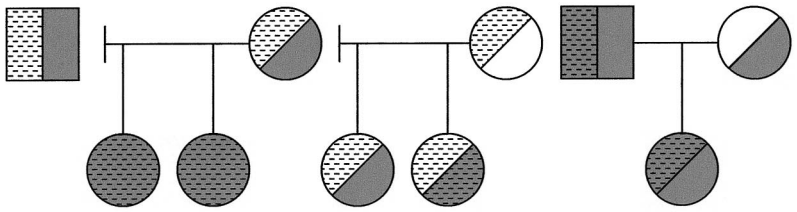
Among the animals tested for these changes, we found fertile animals homozygous for *GDF9* G4 and G7 polymorphisms and the *BMP15* B1 leucine deletion, and conclude that none of those changes result in disruption of the genes sufficient to cause sterility. We also found animals that were heterozygous for *GDF9* and *BMP15* mutations together (e.g., daughter 997552; Fig. 4) and these animals were fertile.

We propose the following nomenclature for the three mutations associated with the sterility phenotype based on the current names for known fertility genes in sheep [19, 36]: $FecX^G$ for *BMP15* B2 and $FecX^B$ for *BMP15* B4 (*X*, X-chromosome; ^{G}, Galway; ^{B}, Belclare), and $FecG^H$ for the *GDF9* G8 mutation (*G*, *GDF9*; ^{H}, high fertility). Genotypic data for these three mutations in all sterile ewes is summarized in Table 3. The genotypes described above explain 35 out of 36 Belclare cases and 26 out of 30 Cambridge cases. All ewes that were homozygous for $FecG^H$ (G8), $FecX^G$ (B2), or $FecX^B$ (B4) were sterile. Also, no fertile ewes were found that were $FecX^G/FecX^B$. There were 45



Animal		R830	930458	930459	97044	930811	930812	87833	930810	948302	78100
Phenotype Sterile (S) Fertile (F)		n/a	S	S	F	S	S	F	F	F	F
GENOTYPE	B2	+/Y	B2/+	B2/+	B2/+	+/+	+/+	+/+	+/+	+/+	+/+
	B4	B4/Y	B4/+	B4/+	+/+	B4/+	B4/+	+/+	B4/+	B4/+	+/+
	G8	G8/+	G8/+	+/+	+/+	G8/G8	G8/G8	G8/+	+/+	+/+	+/+

FIG. 3. Schematic representation of genotypes within F700-Belclare pedigree. The pedigree represents sire R830 mated to three ewes (9704, 8783, and 7810) and their six female offspring. The table below the pedigree diagram shows the phenotype and genotypes for each of the animals. The phenotype of each animal is indicated as sterile (S), fertile (F), or not applicable (n/a) for the male. Horizontal dashed lines within the circles (females) or squares (males) denote carriers of the *BMP15* B2 ($FecX^G$) mutation, diagonal lines denote carriers of the *BMP15* B4 ($FecX^B$) mutation, and the solid gray denotes carriers of the *GDF9* G8 ($FecG^H$) mutation. Half circles or half squares denote those animals heterozygous for a mutation. Sire R830 is heterozygous for G8 ($FecG^H$) (gray) but hemizygous for B4 ($FecX^B$) as *BMP15* is carried on the X chromosome (Y denotes the Y chromosome).



Animal	962101	962152	976234	962152	976234	976234	976234	930142	930142	8874
Phenotype Sterile (S) Fertile (F)	n/a	S	S	F	F	S	F	n/a	S	F
GENOTYPE B2	B2/Y	B2/B2	B2/B2	B2/+	B2/+	B2/B2	B2/+	B2/Y	B2/+	+/+
GENOTYPE B4	+/Y	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
GENOTYPE G8	G8/+	G8/G8	G8/G8	G8/+	G8/+	G8/+	+/+	G8/G8	G8/G8	G8/+

FIG. 4. Schematic representation of genotypes within Cambridge pedigrees. The pedigree represents sire 962101 mated to two ewes (962152 and 976234) and their four female offspring, and sire 930142 mated to ewe 8874 and their one female offspring. The table below the pedigree diagram shows the phenotype and genotype for each of the animals. The phenotype of each animal is indicated as sterile (S), fertile (F), or not applicable (n/a) for the male. Horizontal dashed lines within the circles (females) or squares (males) denote carriers of the *BMP15* B2 (*FecX*^{G}) mutation and the solid gray denotes carriers of the *GDF9* G8 (*FecG*^{H}) mutation. Dashed gray squares and circles denote carriers of both mutations. Half-and-half shading denotes those animals heterozygous for the mutations. Sire 962101 is heterozygous for G8 (*FecG*^{H}) (gray) but hemizygous for B4 (*FecX*^{G}) as *BMP15* is carried on the X chromosome (Y denotes the Y chromosome).

fertile Cambridge ewes that were heterozygous for mutations in both *GDF9* and *BMP15* (*FecX*^{G}/*FecX*$^{+}$; *FecG*^{H}/*FecG*$^{+}$), and six fertile Belclare ewes (*FecX*^{B}/*FecX*$^{+}$; *FecG*^{H}/*FecG*$^{+}$). However, we also observed five sterile ewes (four Cambridge and one Belclare) that could not be explained by the genotypes for *BMP15* and *GDF9* (Table 3). The entire coding region of both the *GDF9* gene and the *BMP15* gene has been sequenced for these Cambridge animals and no additional polymorphisms have been found.

Heterozygous Animals Have Increased Ovulation Rate

Cambridge and F700-Belclare sheep have increased ovulation rates as well as infertility [9, 22]. We examined ovulation rate data that were available for the purebred fertile ewes that had been genotyped for the *FecX*^{G}, *FecX*^{B}, and *FecG*^{H} mutations (Table 4). All mutations significantly increased ovulation rate. The estimate (±SEM) for the effect of *FecX*^{G} was 0.77 ± 0.537 ($P = 0.16$) in Belclare ewes and 1.18 ± 0.387 ($P < 0.01$) in Cambridge ewes, and for *FecX*^{B} was 2.38 ± 0.549 ($P < 0.001$) in Belclare ewes. The effect of *FecG*^{H} was 1.79 ± 0.548 ($P < 0.01$) in Belclare ewes and 2.35 ± 0.386 ($P < 0.001$) in Cambridge ewes. The test for interaction to determine whether the effect of the *BMP15* mutation is dependent on the genotype at the *GDF9* locus does not appear to be significant. The test for interaction between the effects of BMP15 (*FecX*^{G}) and *GDF9* (*FecG*^{H}) in the Cambridge animals was $P > 0.4$. The statistical test for interaction between *BMP15* (*FecX*^{B}) and *GDF9* (*FecG*^{H}) in the Belclare dataset approached significance ($P = 0.064$) but should be treated with caution due to the very small number of ewes with some of the allele combinations.

Progeny test data provide another body of evidence for the magnitude of the effects of the mutations on ovulation rate. The progeny means for the 10 F700-Belclare rams with known genotypes were used to estimate the effects of

TABLE 3. Genotype of all sterile ewes tested for alleles of *BMP15* (*FecX*) and *GDF9* (*FecG*).

Genotype[a] for		No. of sterile ewes from	
BMP15	GDF9	F700 Belclare	Cambridge
FecX^{G}/*FecX*^{G}	*FecG*$^{+}$/*FecG*$^{+}$	0	9
FecX^{B}/*FecX*^{B}	*FecG*$^{+}$/*FecG*$^{+}$	27	0
FecX^{G}/*FecX*^{B}	*FecG*$^{+}$/*FecG*$^{+}$	6	0
FecX^{+}/*FecX*$^{+}$	*FecG*^{H}/*FecG*^{H}	2	14
FecX^{G}/*FecX*^{G}	*FecG*^{H}/*FecG*^{H}	0	3
FecX^{+}/*FecX*$^{+}$	*FecG*$^{+}$/*FecG*$^{+}$	0	2[b]
FecX^{G}/*FecX*$^{+}$	*FecG*^{H}/*FecG*$^{+}$	0	1[b]
FecX^{B}/*FecX*$^{+}$	*FecG*^{H}/*FecG*$^{+}$	1[b]	0
FecX^{+}/*FecX*$^{+}$	*FecG*^{H}/*FecG*$^{+}$	0	1[b]

[a] A^{+} denotes wild-type allele.
[b] Unexplained by the mutations in *BMP15* or *GDF9*.

TABLE 4. Least squares means for ovulation rate of Belclare and Cambridge ewes according to genotype for *BMP15* (*FecX*) and *GDF9* (*FecG*).

Genotype[a] for		Breed[b]	
BMP15	GDF9	F700 Belclare	Cambridge
FecX^{+}/*FecX*$^{+}$	*FecG*$^{+}$/FecG^{+}	1.92 ± 0.28 (n = 11)	2.27 ± 0.49 (n = 10)
FecX^{G}/*FecX*$^{+}$	*FecG*$^{+}$/*FecG*$^{+}$	2.69 ± 0.48 (n = 4)	3.11 ± 0.44 (n = 15)
FecX^{B}/*FecX*$^{+}$	*FecG*$^{+}$/*FecG*$^{+}$	3.26 ± 0.18 (n = 32)	—
FecX^{+}/*FecX*$^{+}$	*FecG*^{H}/*FecG*$^{+}$	2.67 ± 0.89 (n = 1)	4.28 ± 0.31 (n = 28)
FecX^{G}/*FecX*$^{+}$	*FecG*^{H}/*FecG*$^{+}$	—	5.80 ± 0.27 (n = 38)
FecX^{B}/FecX^{+}	*FecG*^{H}/*FecG*$^{+}$	6.09 ± 0.55 (n = 3)	—

[a] A^{+} denotes wild-type allele; the effect of one copy of *FecG*^{H}, *FecX*^{B}, *or FecX*^{G} was significant ($P < 0.01$) in all cases except for *FecX*^{G} in Belclare ($P = 0.16$).
[b] Numbers in parentheses equal number of ewes; a total of 653 ovulation rate records were available for Cambridge and 430 records for Belclare.

each mutation. The data were analyzed separately for ewe lambs and adult ewes. The resulting estimates (± SEM) for the effect of $FecX^G$ were 0.62 ± 0.16 (lambs; $P < 0.01$) and 0.72 ± 0.15 (ewes; $P < 0.001$); for $FecX^B$, they were 0.76 ± 0.09 (lambs; $P < 0.001$) and 1.11 ± 0.09 (ewes; $P < 0.001$); and for $FecG^H$, the estimates were 0.83 ± 0.16 (lambs; $P < 0.001$) and 1.75 ± 0.16 (ewes; $P < 0.001$). The effects were significant in all cases and there was no evidence for any interaction between the effects of *BMP15* ($FecX^B$) and *GDF9* ($FecG^H$) ($P > 0.7$ for lambs and $P > 0.5$ for ewes).

The weighted estimates, based on all of the results from both breeds, for the effects on ovulation rate were, for $FecX^G$, 0.70 ± 0.105; for $FecX^B$, 0.97 ± 0.063; and for $FecG^H$, 1.39 ± 0.107. The effects of the two *FecX* mutations can be compared directly in the Belclare breed, and on this basis, the $FecX^B$-$FecX^G$ contrast was 0.28 ± 0.097 ($P < 0.05$).

DISCUSSION

The present study reports three new nucleotide polymorphisms in the sheep *BMP15* gene, two of which disrupt the protein product. One of those disrupting the protein causes premature termination and the other produces a nonconservative amino acid change in the mature coding region of the peptide. Both of these mutations are associated with sterility and increased ovulation rate in sheep. We also report eight new polymorphisms in the sheep *GDF9* gene, three of which change amino acids in the mature coding region of the protein. One of these produces a nonconservative change and is associated with sterility and increased ovulation rate.

The genes *GDF9* and *BMP15* both code for distinct proteins that are members of the transforming growth factor beta (TGFβ) superfamily. In the ovary, GDF9 and BMP15 have now been shown to be expressed exclusively in the developing oocyte in humans [37], rodents [26, 28, 38], ruminants [19, 31, 39], and marsupials [40]. In sheep, expression of GDF9 can be seen in primordial follicles whereas BMP15 is expressed in primary follicles [19, 31].

This study confirms previous observations that, in sheep, BMP15 is essential for folliculogenesis. Females carrying two copies of inactivating mutations are sterile [19] and anovulation can be induced by immunizing sheep with BMP15 peptide [41]. It also provides additional evidence that heterozygotes carrying mutations in one copy of *BMP15* have an increased ovulation rate. The two new *BMP15* mutations described here bring to four the total number of naturally occurring *BMP15* mutations that have been reported in populations that have been selected for high ovulation rate. Heterozygous carriers of the new mutations in *BMP15* (either $FecX^G$ or $FecX^B$) show increased ovulation rate of a magnitude similar to that seen in Inverdale ($FecX^I$) and Hanna ($FecX^H$) sheep [20]. However the evidence suggests that the effect of $FecX^B$ is greater than the effect of $FecX^G$. This will need to be confirmed by further evaluation studies.

Our present results show that GDF9 is also essential for folliculogenesis in sheep. Animals with two copies of the $FecG^H$ mutation are infertile. This observation supports GDF9 immunization data in sheep [41], which show that normal follicle development is arrested at the type 1a and type 2 stages in sheep immunized with GDF9-specific peptide. Our results also show, for the first time, that sheep heterozygous for the *GDF9* $FecG^H$ mutation have an increased ovulation rate and there is no indication that the magnitude of the effect is breed dependent. In addition, the effect of the *GDF9* mutation is significantly greater than the effect of the *BMP15* mutations. Animals heterozygous for both a *BMP15* mutation and the *GDF9* mutation have an even higher ovulation rate. The evidence indicates that the effects on ovulation rate of one copy of a *BMP15* mutation and one copy of the *GDF9* are additive and supports the view that the biological effects of BMP15 and GDF9 are distinct [42].

Increased ovulation rates have not been observed in heterozygous mice with knockouts of either *BMP15* or *GDF9* [29]. Double knockouts of both *GDF9* and *BMP15* in mice are infertile with a similar phenotype to $GDF9^{-/-}$ mice alone, but *BMP15* knockout mice with one active copy of *GDF9* have a lower fecundity than $BMP15^{-/-}$ females [29], suggesting that the relative dose of these gene products may also play a role in mice. A study of the aberrant follicle development in women with polycystic ovary syndrome (PCOS) has shown delayed and reduced GDF9 expression, suggesting that GDF9 dysfunction is contributing to PCOS [43].

Most members of the TGFβ superfamily are biologically active as dimers, and although GDF9 and BMP15 do not contain the cysteine residue responsible for covalent interchain disulphide bonding seen in other members of the family, these molecules are also thought to be biologically active as dimers. However, it is not known whether the physiologically active dimers are homodimers (GDF9-GDF9 and BMP15-BMP15), or heterodimers (GDF9-BMP15), whether all three dimer forms play a role, or whether different combinations are predominant in different species. It is still unclear whether GDF9 and BMP15 act separately or together in sheep, but our observations, that the effects of a *GDF9* mutation and a *BMP15* mutation together in one animal appear to be additive, imply that GDF9 and BMP15 are likely to be working independently.

The structures of BMP15 or GDF9 have not yet been determined, and their receptors have not been fully determined, although recent evidence suggests that the type-II receptor BMPRII interacts with both BMP15 and GDF9, and that the type-I receptor BMPR1B (ALK6) interacts with BMP15 [44, 45]. However, previous experimental and structural data for other members of the TGFβ superfamily provide information about the likely effects of each of the three mutations ($FecX^G$, $FecX^B$, and $FecG^H$) on the biological activity of GDF9 and BMP15 and help to explain the association with sterility.

The $FecX^G$ mutation results in premature termination of BMP15 protein before the mature active protein-processing site. This mutation would result in no mature protein being translated, and is even earlier in the gene than the Hanna ($FecX^H$) premature termination mutation [19], which results in infertility in sheep. Single amino acid changes, which do not introduce such a severe effect as premature truncation, also clearly affect BMP15 function. Thus, Inverdale ($FecX^I$) sheep have a distinct T to A mutation, which substitutes valine (conserved across most TGFβ superfamily members) for aspartic acid at residue 31 of the mature BMP15 peptide. Modeling of that substitution (changing a hydrophobic valine with a negatively charged aspartate) indicated that it results in a change in the electrostatic surface potentials of an area involved in dimer formation and appears to disrupt dimerization and hence abolish biological activity [19]. The *BMP15* $FecX^B$ mutation changes an uncharged polar serine residue (residue 99 of mature BMP15), which is also conserved across most members of the TGFβ superfamily, to

a nonpolar isoleucine. This serine (and the nearby conserved leucine) has been shown to be essential for receptor binding to the type II receptor by structural determination of ligand-receptor complexes with BMP2, BMP7, and activin A, and by site-directed mutagenesis [46–48]. In F700-Belclare sheep, it appears that this mutation abolishes biological activity of BMP15, presumably by affecting receptor binding.

The *GDF9 FecG^H* mutation changes an uncharged polar serine residue (residue 77 of mature GDF9) to a nonpolar phenylalanine in a region of the molecule that is likely to be involved in binding to the type I receptor [49]. Therefore, this mutation could be affecting the ability of the ligand to bind to a receptor. However, this change also occurs only three residues away from a conserved histidine of the mature GDF9 peptide. In BMP7, this conserved histidine exhibits hydrogen bonding to three residues of the paired molecule in the BMP7 dimer [50] and TGFβ3 [51]. GDF9 and BMP15 lack the interchain disulphide bond that forms a covalent link between monomers of the biologically active dimer in most other members of the TGFβ superfamily. Thus, it may be that, in GDF9, the hydrogen bonds between monomers are even more critical for maintaining dimer stability and the *GDF9 FecG^H* mutation could be affecting biological activity by disrupting dimerization.

The sterility phenotypes of four Cambridge animals and one Belclare animal remain unexplained by the mutations described here. The Belclare case was unusual in that, while one ovary was noted as small and without any follicles at three independent examinations, the other ovary was normal in size and had some small follicles on one occasion; the uterus (which is typically infantile in cases classified as sterile) was of normal size. However, the sterility in the four unexplained Cambridge animals may well be due to a new specific mutation because they all had a definite sterile phenotype and were related through one male on both sides of the pedigree. Such a mutation may be in a gene that induces similar phenotypic effects, possibly by disrupting the function of GDF9 or BMP15. This could include mutations in the promoter regions for these genes or their receptors.

These findings provide the first evidence that mutations in *GDF9* and *BMP15* are associated with the reproductive effects seen in the Cambridge and F700-Belclare breeds of sheep. The increased ovulation rate and the majority of the sterility phenotypes in these animals can be explained by the presence of heterozygous mutations and homozygous mutations, respectively, in these genes. While previous descriptions of the Cambridge breed assumed an autosomal mode of inheritance [5], subsequent findings led to the hypothesis that two loci were required to explain the variation seen in both the Cambridge and Belclare populations [9, 22]. The present results confirm this. The X-linked effects of the *BMP15* (*FecX^G* and *FecX^B*) mutations were masked by segregation of the autosomal *GDF9* (*FecG^H*) mutation. Ongoing investigations into the basis of the unexplained sterile phenotypes are likely to reveal further insights into the events controlling follicle and oocyte development.

ACKNOWLEDGMENTS

The authors thank Eithne Powell and Bart Crowley for assistance with DNA analysis, Janine Rallières for SSCP genotyping, and Pat Madden, Tom Lally, and Anne Curley for technical support with ovulation rate measurements, flock management, and data analysis.

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