Homozygosity for a single base-pair mutation in the oocyte-specific GDF9 gene results in sterility in Thoka sheep

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Abstract

The control of fecundity is critical in determining mammalian offspring survival. It is regulated principally by the ovulation rate, so that primates and large farm species commonly have a single offspring. Previously, several mutations have been identified in sheep which increase the naturally low ovulation rate; although in some cases homozygous ewes are infertile. In the present study we present a detailed characterization of a novel mutation in growth differentiation factor 9 (GDF9), found in Icelandic Thoka sheep. This mutation is a single base change (A1279C) resulting in a nonconservative amino acid change (S109R) in the C-terminus of the mature GDF9 protein, which is normally expressed in oocytes at all stages of development. Genotyping all animals for which reproductive records were available confirmed this mutation to be associated with increased fecundity in heterozygous ewes and infertility in homozygotes. Analysis of homozygote ovarian morphology and a number of genes normally activated in growing follicles showed that GDF9 was not involved in oocyte activation, but in subsequent development of the follicle. This study highlights the importance of oocyte factors in regulating fertility and provides new information for structural analysis and investigation of the potentially important sites of dimerization or translational modifications required to produce biologically active GDF9. It also provides the basis for the utilization of these animals to enhance sheep production.


Introduction

In livestock species, ovulation rate and subsequent embryo survival are traits of high economic importance, with increased numbers of offspring born alive being advantageous for efficient livestock production with a reduced environmental footprint. As a result of studies in sheep, a species having close parallels to humans in the regulation of ovarian function throughout life, major insights have been gained through investigation of the effects of naturally occurring gene mutations that not only increase ovulation rates, but, in two cases, also contribute to sterility phenotypes (Campbell et al. 2003, McNatty et al. 2005). Mutation of the bone morphogenetic protein receptor (BMPR) IB gene (the Booroola mutation) results in increased ovulation rate in both heterozygous and homozygous ewes (Mulsant et al. 2001, Souza et al. 2001, Wilson et al. 2001). In contrast, mutations in oocyte-specific BMP15 (FecX) increase ovulation rate by about 1.0 in heterozygous carriers, but result in infertile ‘streak’ ovaries in homozygotes (Davis et al. 1991, 1992, Hanrahan et al. 2004, Martinez-Royo et al. 2008, Monteagudo et al. 2009). Finally, a mutation in the oocyte-specific growth differentiation factor 9 (GDF9) gene is also associated with increased ovulation rate in heterozygous ewes, but sterility in homozygotes (Hanrahan et al. 2004). If managed appropriately, ewes with these mutations are of value to the farming industry. However, their exploitation is only achieved by the capacity to identify animals carrying the relevant mutation and this, combined with an understanding of the mechanisms of action, contributes directly to our understanding of the control of follicle development.

In the current study, we report the identification and characterization of a novel mutation in the ovine GDF9 gene, which also results in heterozygote fecundity, but homozygote sterility. This mutation is believed to have originated in Icelandic sheep, where all multiple births on one farm could be traced back to a ewe named Thoka (Jónmundsson & Adalsteinsson 1985, Adalsteinsson et al. 1989). The so-called ‘Thoka’ gene was introduced...
into the UK and has now been established in a Cheviot sheep flock (King et al. 1990, Russel et al. 1997). Thoka Cheviots are phenotypically indistinguishable from pure-bred Cheviots in all traits apart from litter size (Rhind et al. 2000), where the Thoka mutation is estimated to result in ~0.6 more lambs per ewe lambing in heterozygous animals (Walling et al. 2002). This paper identifies the causative mutation and presents comprehensive evidence for its effects on ovarian function. We have used a combination of state-of-the-art statistical techniques and bioinformatics, with morphological, endocrinological, and histological analyses, to derive results that would not be possible without recourse to such a multidisciplinary approach.

Results

Demonstrating the presence of a gene affecting fecundity

Litter size is a complex and poorly-repeatable phenotype, and complex segregation analyses (Morton & MacLean 1974) are required to determine whether differences between animals are dominated by one gene with a large effect. We used MCMC methods (Guo & Thompson 1992), implemented through a Gibbs Sampler, to apply a complex segregation analysis to the litter size data (see Walling et al. 2002 for preliminary results). The analyses assumed the mixed inheritance model combining known fixed effects, polygenic effects influencing the trait, as well as major gene effects. Full details are given in Materials and Methods.

The major gene was initially modeled as an additive autosomal biallelic locus with Mendelian transmission probabilities. However, with increasing evidence that ewes homozygous for the putative mutation (TT) were infertile, analyses were rerun with the restriction that if a ewe had an observed litter size then she could not have a TT genotype. Results are presented for the latter case but, due to the low frequency of potential TT ewes in the dataset, most inferences from these two analyses are the same. A further restriction made was that foundation Cheviot ewes were wild-type noncarriers (+ +). The dataset analyzed contained 2089 litter size records from 694 ewes (having 1–7 parities), with 1666 animals in the pedigree.

The marginal posterior distribution for the effect of the putative Thoka mutation is shown in Fig. 1. The mean of this distribution is 0.613, the S.D. is 0.052 (this is analogous to the frequentist S.E.M.) and the 95% posterior density region is 0.509–0.718. Hence, there is overwhelming evidence that there is a mutation segregating in this population that influences litter size and the effect is ~0.6 lambs per litter. The population's mean litter size is 1.87 and the polygenic heritability is 0.17. From the genotype probabilities provided by the analysis a ‘Thoka index’ (Index = Probability (T+) + 2 × Probability (TT)) was calculated and subsequently used for flock management purposes and to assist in finding the chromosome location of the putative gene.

Mapping the chromosomal location of the fecundity gene to the GDF9 region of chromosome 5

A genome scan was undertaken to identify the chromosomal location of the statistically-inferred fecundity gene. A total of 81 microsatellite markers, spaced throughout the sheep autosomes, were chosen from the sheep linkage map (Maddox et al. 2001). These markers were then genotyped for 334 animals for which DNA was available. The software algorithm Mendelsoft (Sanchez et al. 2008) was used to resolve genotype inconsistencies between related animals using a maximum likelihood approach. Quantitative trait locus (QTL) mapping was performed using a two-step variance component interval-mapping procedure (George et al. 2000) appropriate for complex pedigrees with missing marker information. Firstly, the proportion of genes identical-by-descent (IBD) between all individuals is estimated at each chromosomal location; then, the contribution of the chromosomal location to the phenotypic variance is assessed using residual maximum likelihood (REML). Full details of the markers and the analyses are given in the Materials and Methods.

A significant (P < 0.05) QTL was observed on chromosome 5 and likelihood ratio test (LRT) statistic profiles are shown for litter size and Index, for which trait the evidence of a QTL was even stronger (Fig. 2). The significance threshold was determined using a chi-square)}
distribution. In both cases, profiles maximized close to the GDF9 locus, suggesting GDF9 as an obvious candidate due to its previous implication as a gene with a major effect on litter size (Davis 2005). The polygenic heritability at the point of maximum evidence for a QTL was 0.17, with the QTL heritability being 0.16.

Reproductive tract abnormalities and infertility correlate with predicted homozygosity for the Thoka mutation

Ewes that had shown repeated infertility, and which were predicted by the segregation analyses to have a possibility of being homozygous for the putative Thoka mutation, were found at post-mortem to have severe reproductive tract abnormalities. Uteri were rudimentary and ovaries were small and inactive, containing no preovulatory follicles or corpora lutea (typical ‘streak’ ovaries; Fig. 3B). A normal reproductive tract from a wild-type animal is shown in Fig. 3A for comparison.

GDF9 sequencing

The GDF9 gene was proposed as an obvious candidate for the Thoka phenotype. Therefore, the coding region for GDF9 was sequenced in five of the infertile ewes with reproductive tract abnormalities identified above, and which had been predicted by the segregation analyses to be potentially homozygous for the mutation (TT). Additionally, nine unrelated non-Thoka carrying Cheviot ewes were sequenced for the GDF9 coding region. All the infertile ewes were homozygous for one single nucleotide polymorphism (SNP), a substitution (A→C) at coding base 1279 (Fig. 4A). All unrelated ewes were homozygous for the wild-type. This SNP affected amino acid residue 427, corresponding to residue 109 within the mature coding sequence (Panther classification system; Sorting Intolerant From Tolerant (SIFT) analysis (Ng & Henikoff 2006)). The region containing this SNP was sequenced in genomic DNA from a further five animals with abnormal reproductive tracts and all had the same mutation. The entire coding region in two putative heterozygous Thoka ewes was also sequenced and both A and C were identified at the location of the A1279C point mutation.

A further five polymorphisms were detected, but only in ewes lacking, or heterozygous for, the polymorphism described above. Of these, four have previously been reported (G3, G4, G5, G6; Hanrahan et al. 2004) and do not contribute to a sterility phenotype. One ewe had a single copy of a G→A substitution at coding base 750 which did not change the corresponding amino acid.
Population-level confirmation of the GDF9 SNP effect

To verify that the identified A→C SNP at coding base 1279 of GDF9 is, beyond reasonable doubt, the causative mutation, 390 ewes from our population with litter size data were genotyped for the SNP. Using a Bayesian mixed model association analysis with data augmentation through genotype inference (Pong-Wong & Woolliams 1996; see Materials and Methods for a full description) we verified an association between the SNP and litter size. The mean of the marginal posterior distribution for the effect of the SNP (Fig. 1) was 0.682, the S.D. was 0.043 and the 95% posterior density region was 0.597–0.765. Therefore, the SNP effect on litter size is slightly larger and more precisely estimated than that inferred from the segregation analysis, as errors in predicting genotypes will be largely avoided. Furthermore, the genotype probabilities suggest that the original Icelandic sires were the source of the SNP in this population.

Further statistical verification was achieved using QTL variance component analyses. The model was the same as described above, except that the dataset was reduced to animals having both microsatellite and SNP genotype data (659 records, 561 animals in pedigree, 210 animals with records and genotypes), and the analyses were performed with and without the SNP fitted as a fixed effect. Ignoring the SNP, the QTL profile (Fig. 5) showed marginally stronger evidence of a QTL than was seen for the full dataset (Fig. 2), with the peak directly above the GDF9 locus. However, when the SNP effect was fitted as a fixed effect all evidence for QTL at this location disappeared (Fig. 5). The SNP effect estimated from this analysis was 0.56 ± 0.06. Analyses on the Thoka Index also showed the QTL disappearing when the SNP effect was fitted (results not shown).

These results provide overwhelming statistical evidence for the SNP being either the causative mutation or in tight linkage disequilibrium with the causative mutation. Together with infertile ewes being homozygous for the SNP, the lack of other SNPs in the genotyped region that could plausibly be the causative mutation, and the predicted consequences of this

Figure 4 Thoka GDF9 sequencing and mutation detection. (A) Nucleotide substitution (A→C) for the Thoka GDF9 (FecTT) mutation. (B) Predicted amino acid sequence of sheep GDF9 protein. The numbers at the start of each line indicate amino acid positions in the full-length unprocessed protein. The RRHR furin protease cleavage site and predicted start of mature protein is underlined. The position of the Thoka GDF9 S427R (S109R in mature peptide) mutation associated with the sterility phenotype is highlighted in gray. The position of the previously reported GDF9 S395F (S77F in mature peptide; Hanrahan et al. 2004) mutation is indicated by the white box.

Figure 5 QTL profiles on ovine chromosome 5, using a reduced data set containing only ewes with microsatellite and SNP genotypes for litter for situations where the SNP genotype is not fitted and where it is fitted as a cofactor. The vertical axis shows −2 ln(L0−L1), where L1 is the log-likelihood of the model including the QTL effect and L0 is the log-likelihood without the QTL effect. The 5% significance threshold is shown by the horizontal line. The horizontal axis shows the name and position (cM) of the genotyped markers. All evidence for a QTL disappears when the GDF9 SNP effect is accounted for.
SNP on GDF9 protein structure and function, there is evidence beyond reasonable doubt that this SNP is the causative mutation.

**Characterization of the Thoka phenotype: ovarian morphology**

The cortical region of ovaries from TT ewes (n=5) contained large numbers of primordial follicles, as well as follicles with up to two layers of granulosa cells, many of which appeared abnormal (Fig. 6A). Follicles with two to four layers of asymmetrically arranged granulosa cells were occasionally observed. A typical section from a + + ovary, containing follicles at several stages of development and with fewer primordial follicles within the ovarian cortex, is shown for comparison (Fig. 6E). Some TT follicles contained oocytes which had developed to a size equivalent to that seen in normal antral follicles. At higher magnification it was evident that many follicles appeared either to have totally collapsed or to be in the process of collapsing (Figs 6B, 9C, E and G and 10). Other abnormalities observed were occasional clusters of oocytes, surrounded by a layer of granulosa cells (Fig. 6C), and nodular structures of varying sizes made up of granulosa-like cells (Figs 6D and 9A), both apparently enclosed within a basement membrane identified by immunohistochemistry for laminin (see below). The ‘nodules’ were generally found in the inner cortex and medulla of the ovary. Serial sections confirmed the absence of oocytes within these structures (Supplementary Figure 1, see section on supplementary data given at the end of this article).

**TT follicles are deficient in proliferating granulosa cells**

5-Bromo-2′-deoxyuridine (BrdU) incorporation and subsequent immunohistochemistry were used to assess the extent of ovarian cell proliferation in three TT and two + + ewes. Occasionally, an isolated cell within TT follicles was labeled (Fig. 7A), but generally there was little evidence for cell proliferation, even though some oocytes had clearly activated as indicated by increases in size of up to 80 μM and production of a zona pellucida (ZP; Fig. 7B). BrdU incorporation was confirmed by immunostaining on spleen sections (data not shown). In contrast, + + follicles which had developed beyond the primary stage, with enlarged oocytes up to 80 μM in diameter, contained many proliferating granulosa and theca cells (Fig. 7C). As expected, granulosa cells in + + primary follicles, with small oocytes 20–30 μM in diameter and little or no ZP development, did not incorporate BrdU and thus...
did not appear to be actively proliferating at this stage (Fig. 7D). Similar results were obtained by immuno-histochemistry for both Ki67 and phospho-H3 as markers of mitosis (results not shown). There was no evidence for increased cell death in TT ovaries. Cleaved caspase 3 immunostaining detected apoptotic cells in wild-type follicles, but not in TT follicles (data not shown).

Characterization of the Thoka phenotype: endocrinology

Plasma FSH and LH were measured during the luteal and follicular stages of the estrous cycle (+ +, n = 9) and at corresponding time points in noncyclic animals (TT, n = 5). Levels of both hormones were higher (P < 0.01 and P < 0.05 respectively; Fig. 8C and D) in TT ewes. As expected, plasma progesterone levels increased during the luteal phase of the estrous cycle in + + ewes, but remained basal in correspondingly timed samples from TT ewes, confirming the lack of ovulation and corpus luteum formation (data not shown).

Characterization of the Thoka phenotype: histology

To assess developmental competence of follicles and oocytes in TT ewes, compared with + + animals, ovary sections were immunostained using antibodies against laminin, mouse vasa homologue (MVH), deleted in azoospermia (DAZL) and ZP protein 3 (ZP3). Laminin staining confirmed the presence of a basal membrane surrounding follicles at all stages present in TT and + + ovaries (Fig. 9A and B). The nodular structures described above, containing granulosa-like cells, but lacking oocytes, were also clearly surrounded by a basement membrane (Fig. 9A). MVH and DAZL were expressed at high levels in oocytes from all follicle stages in + + ovaries and in oocytes from the majority of TT follicles (Fig. 9C–F). However, expression of both proteins was reduced or absent in abnormal TT oocytes that appeared to be either in the process of collapsing and degenerating or that had already collapsed (Fig. 9C and E). The ZP, identified by ZP3, was present around oocytes in follicles from secondary stage onwards in + + animals (Fig. 9H), and was present around larger oocytes in TT follicles (Fig. 9G). Interestingly, a thicker area of ZP was often observed in collapsing or collapsed oocytes.

Progression of TT follicle development and degeneration

A proposed scheme for the progression of TT follicle development and subsequent degeneration highlighted by ZP3 staining of the ZP is presented in Fig. 10. Images A–D show the development of ZP around oocytes of increasing size. Image E demonstrates increased thickness of the ZP layer in an oocyte which appears to be beginning to degenerate, and images F–I apparently show the gradual oocyte collapse as degeneration proceeds, until all that remains is the ZP3 positive ZP with associated granulosa cells in a nodule.

Discussion

The studies reported here detail the multidisciplinary, cooperative approach that is typical of studies that detect causative mutations underlying QTL. Nevertheless, it is a rare example of moving from population-level phenotypic observation to causative mutation for a
complex trait. Previous examples in livestock include the Booroola mutation for increased fecundity in sheep (Mulsant et al. 2001, Souza et al. 2001, Wilson et al. 2001), a mutation in the bovine diacylglycerol acyltransferase 1 (DGAT1) gene leading to major effects on milk yield and composition (Grisart et al. 2002), a regulatory mutation in insulin-like growth factor 2 (IGF2) leading to increased muscle growth in pigs (Van Laere et al. 2003) and a mutation in the myostatin gene which creates a microRNA target site and leads to the extreme muscularity observed in Texel sheep (Clop et al. 2006). However, this paper is unique in that the complete process of QTL mapping, detection of the likely causative mutation for a complex trait and investigation of the phenotypic consequences were achieved in one study. With the availability of dense SNP arrays for all major livestock species such examples will become more common. Already, these approaches have enabled rapid detection of mutations causing recessive disorders in cattle (Charlier et al. 2008).

Figure 8 Plasma concentrations of FSH, LH, inhibin A, and estradiol. (A) Changes in plasma levels of FSH in individual animals during the luteal (days 6–11; daily bleeds) and follicular (24–30 h post PG; hourly bleeds) stages of the estrous cycle in cycling ewes, and at the same time points in noncyclic animals. Homozygous Thoka ewes (TT; n = 5) had higher (P < 0.001) levels of FSH compared with wild-type ewes (+ +; n = 9). (B) Changes in plasma levels of LH in individual animals during the luteal (days 6–11; daily bleeds) and follicular (24–30 h post PG; 10 min bleeds) stages of the estrous cycle in cycling ewes, and at the same time points in noncyclic animals. TT ewes (n = 5) had higher (P < 0.001) levels of LH compared with + + controls (n = 9). (C) Mean plasma levels of inhibin A (from 2 samples, one hour apart) during the follicular phase of the estrous cycle and at the same time points in noncyclic animals. TT ewes (n = 5) had lower levels (P < 0.01) of inhibin A compared with + + controls (n = 9). (D) Mean plasma levels of estradiol (from two samples, one hour apart) during the follicular phase of the estrous cycle and at the same time points in noncyclic animals. TT ewes (n = 5) had lower levels of estradiol (P < 0.05) compared with + + controls (n = 9).

However, complex traits under polygenic control will remain challenging.

In the present study, the causative mutation in Thoka ewes (FecTT) was found to be in GDF9, a member of the transforming growth factor β superfamily expressed exclusively in oocytes at all stages of development, including the quiescent primordial follicle stage. The GDF9 mutation reported here produced a nonconservative amino acid change at position 109 within the mature coding region (S109R) that is predicted to affect the function of the protein. This novel mutation is different from a previously reported mutation in the GDF9 gene in sheep (Belclare & Cambridge; FecGH), which produced a nonconservative amino acid substitution at position 77 of the mature protein (S77F; Hanrahan et al. 2004). This mutation also resulted in homozygote sterility and heterozygote fecundity although the effects of our present mutation on follicle development appear to be more severe. Our results are consistent with previous findings, in both sheep (Hanrahan et al. 2004) and...
knockout mice (Dong et al. 1996), that GDF9 is required for folliculogenesis, in particular for the primary to preantral transition.

Ovaries from our homozygous TT ewes were characterized by having very large numbers of primordial follicles, numerous primary stage follicles with a single layer of granulosa cells, and minimal numbers of follicles which had developed beyond the primary stage. However, in no case was there formation of a thecal cell layer and most of the follicles beyond the primordial stage were abnormal. There was no evidence for either active cell proliferation or increased cell death in primary follicles. However, oocyte activation had occurred in TT follicles since large oocytes equivalent to those seen in mature antral follicles in T+ and ++ ovaries were frequent, and immunological analysis indicated that oocyte protein expression, ZP formation and basement membrane formation were normal. In contrast to our present results, the ovaries of the previously reported infertile GDF9 S77F FecGH homozygous ewes contained some follicles which had developed as far as the antral stage including the formation of a thecal cell layer, although the majority of these follicles were abnormal (Hanrahan et al. 2004, McNatty et al. 2005). This suggests that the present S109R mutation has had a greater effect on the production of active GDF9 than the previous mutation, with a phenotype equivalent to that of complete immunoneutralization of GDF9 in sheep (Juengel et al. 2002) or in GDF9-null mice (Dong et al. 1996). Consistent with the total lack of normal follicle development, plasma levels of inhibin A and E2 were low, resulting in elevated levels of gonadotropins (McNeilly et al. 2003).

Figure 9 Immunostaining. Sections through ovaries were immunostained using antibodies against laminin, MVH, DAZL, ZP3 to assess developmental competence of Thoka homozygotes in comparison to wild-type controls. (A and B) Laminin. The granulosa cell layer in TT follicles (A) was enclosed within a basal membrane similar to that in ++ follicles (B). (C and D) MVH. This was expressed in oocytes from all follicles in ++ ovaries (D) and also in oocytes from most follicles in TT ovaries, but was reduced or absent in TT follicles which appeared to be in the process of collapsing (C). (E and F) DAZL. This was expressed in oocytes from all follicles in ++ ovaries (F) and also in oocytes from most follicles in TT ovaries, but was reduced or absent in TT follicles which appeared to be in the process of collapsing (E). (G and H) ZP3. This was present around oocytes in follicles from the secondary stage onwards in ++ animals (H) and around larger oocytes in TT follicles and appeared very prominent in collapsing and collapsed TT follicles (G). Scale bar represents 100 μM.
As in TT sheep, any GDF9-null mouse follicles with more than one layer of granulosa cells appeared to be abnormal, none had developed a thecal cell layer, and serum gonadotropin levels were increased (Dong et al. 1996, Elvin et al. 1999). In addition, in GDF9-null mice electron microscopic analysis showed that the oocytes did not produce a normal complement of cortical granules or undergo normal dispersion of intracellular organelles, which remained clustered around the germinal vesicle and had limited meiotic competence (Dong et al. 1996). Similar studies on follicles of TT ewes are underway.

The ovarian phenotype we observed in infertile homozygous TT ewes is similar in some aspects to that observed in Inverdale sheep homozygous for a mutation in the related oocyte-specific BMP15 gene (II; Braw-Tal et al. 1993). However, unlike in some II sheep (Braw-Tal et al. 1993), and GDF9-null mice (Dong et al. 1996), we did not observe any follicular cysts or abnormal tumor-like structures in our homozygous TT ewes up to 5 years of age. In addition to abnormal follicles, we did observe nodular structures composed of granulosa-like cells, but lacking an oocyte, within TT ovaries. Similar nodules were reported previously in II ewes (Braw-Tal et al. 1993) and GDF9-null mice (Elvin et al. 1999). Gene expression analyses on ovaries from II ewes and GDF9-null mice revealed that mRNA expression was similar between the nodules and type 3 (small preantral) follicles. It was concluded that the cells making up these nodules were derived from follicles that had lost their oocytes, and had retained many of the characteristics of granulosa cells (Juengel et al. 2000). In mice at least, these cells appeared to have lost an ability to undergo apoptosis and thus persisted (Elvin et al. 1999). These data support our proposed scheme for TT follicle development and eventual degeneration as depicted in Fig. 10. It is hypothesized that the oocyte, the remains of which are evident in image I, would eventually disappear completely, leaving a nodule containing the granulosa cells remaining from the original follicle as evident from serial sections through such nodules (Supplementary Figure 1).

The similarity in ovarian phenotype between sheep carrying disrupting mutations of either GDF9 or BMP15 supports the concept that both proteins are directly involved in follicle development. In contrast, in mice GDF9 appears to function independently of BMP15, since BMP15-null mice have a limited phenotype associated with slightly reduced fertility, but minimal effect on follicle activation and progression to preovulatory stages of development (Yan et al. 2001). This has implications for understanding how the present TT GDF9 mutation affects GDF9 function.

In sheep, while BMP15 is absent from primordial follicles, both BMP15 and GDF9 are expressed in...
oocytes from the primary stage of development onwards (McNatty et al. 2005). GDF9 is reported to form noncovalent homodimers, and also heterodimers, with BMP15, although the physiological relevance of the heterodimers remains to be established (Liao et al. 2003). Furthermore, although ovine GDF9 and BMP15 are produced as nearly fully processed dimers in 293H cells, in ovine follicular fluid they exist as uncleaved premature proteins (McNatty et al. 2006). In vitro studies, using stably transfected 293T cells, have suggested that mutant BMP15 interferes with BMP15 or GDF9 secretion, whereas mutant GDF9 (S77F) does not interfere with BMP15 or GDF9 secretion unless it is co-expressed with mutant BMP15 to produce an additive reduction in both BMP15 and GDF9 secretion (Liao et al. 2004). More recent studies have emphasized the complex nature of the interactions between GDF9 and BMP15 using recombinant mouse proteins, and indicate there may well be species specificity in these interactions (McIntosh et al. 2008).

It is unclear how the present homozygous GDF9 S109R mutation prevents development of the granulosa cells of the follicle when the oocyte is activated. Given the apparent need for GDF9 homodimerization or interaction of GDF9 with the pro-region of BMP15 to occur (at least in mice (McIntosh et al. 2008)), in order to allow GDF9 to function, the S77F mutation previously reported, which results in initial follicle development to an antral stage (Hanrahan et al. 2004, McNatty et al. 2005) may be in a region that is not involved in dimerization or interaction with BMP15. In contrast, our S109R mutation is closer to the C-terminus of the protein and thus may be more likely to affect dimerization and either secretion of biologically active GDF9, or failure of activation of the GDF9/BMPR2 (ALK5) putative receptor complex.

In conclusion, in the present study, the evaluation of phenotypic effects at the flock/population level through genetic analysis, combined with endocrinological and anatomical analyses, has led to the identification and characterization of a novel mutation in the oocyte-specific protein GDF9. Heterozygous ewes have increased fecundity, but the mechanisms whereby a single abnormal copy of GDF9 causes this to occur remain to be elucidated. In homozygous ewes this mutation results in infertility due to a complete absence of follicle development in spite of apparently normal activation of the oocyte and expression of a number of oocyte specific genes including those involved in ZP formation. The identification of the single amino acid substitution in the present study has provided new information for structural analysis and investigation of the potential important sites of dimerization, translational modifications of GDF9, or sites of interactions with BMP15 required to produce biologically active GDF9.

Materials and Methods

Animals

Sheep flock establishment and maintenance have been described previously (Russel et al. 1997, Walling et al. 2002). Briefly, using frozen semen, in 1985 two Icelandic rams, believed to be carrying the putative Thoka mutation, were mated with Cheviot ewes at The Macaulay Institute’s Sourhope Research Station (Russel et al. 1997). A flock of 50–120 breeding ewes descended from these matings have been maintained. Initially, daughters of the two rams were retained on the basis of having an ovulation rate (determined by laparoscopic examination of ovaries following synchronization of estrus at ~18 months of age) ≥ 2. Subsequently, ewes with a litter size of ≥ 2 in at least two of their first 3 years of breeding were retained. Until 1995 purebred Cheviot rams were used as sires to minimize the contribution of the Icelandic genome, while selection of highly fecund ewes maintained the putative Thoka mutation in the flock. Thereafter, rams were selected from within the flock, on the basis of their dams’ performance, in an attempt to increase the frequency of the putative Thoka mutation, while minimizing inbreeding. Since 2001, complex segregation analyses (see below) have been used to assign probabilities of animals being homozygous wild-type (+/+), heterozygous (T+) or homozygous Thoka (TT), and these probabilities have been used to assist selection of replacement ewes and rams.

Complex segregation analyses and Bayesian association analysis

The mixed inheritance model was:

\[ y = X\beta + Z_1a + Z_2c + Wg + e \]

where \( y \) is a vector of observations (litter size); \( X, Z_1, Z_2 \) are known incidence matrices respectively relating \( \beta, a \) and \( c \) to \( y \); \( W \) is the unknown genotype incidence matrix, calculated for each iteration, relating to \( g \); \( \beta \) is a vector of fixed effects; \( a \) is a vector of random additive polygenic effects (variance \( \sigma_a^2 \)); \( c \) is a vector of permanent random environmental effects; \( e \) is a vector of random residuals (variance \( \sigma_e^2 \)); and \( g \) is a vector of the allelic effects. The variance components were estimated during the analysis.

The conditional distributions needed to sample all parameters and effects in this mixed inheritance model are well established (Guo & Thompson 1992, Wang et al. 1993, 1994, Firat 1995, Janss et al. 1995). Prior distributions were uniform across the parameter space, our previous analysis (Walling et al. 2002) having demonstrated that prior distribution assumptions made little difference to the interpretation of the results. The analyses were implemented using a Gibbs Sampler and results were obtained from a Markov chain of 100 000 realizations sampled every 50 iterations after a burn-in period of 10 000 iterations (i.e. total length of chain = 5 010 000).

The mixed inheritance model used in the Bayesian association analysis was:
\[ y = X\beta + Z_1a + Z_2\gamma + v + e \]

where all terms are defined as above except that \( W^* \) is the known genotype incidence matrix relating to \( g^* \) for genotyped animals, and an unknown genotype incidence matrix, calculated for each iteration, for ungenotyped animals; \( g^* \) is a vector of known allelic effects for genotyped animals and inferred allelic effects for ungenotyped animals. The analyses were implemented using a Gibbs Sampler, as described above.

**QTL analyses**

The microsatellite markers and their chromosomal positions (from the consensus sheep linkage map, http://rubens.its.unimelb.edu.au/~jillm/jill.htm) are described in Supplementary Table 1, see section on supplementary data given at the end of this article. The full statistical model used to describe the QTL effect at each chromosome location was:

\[ y = X\beta + Z_1a + Z_2\gamma + v + e \]

where \( v \) is a vector of additive QTL effects with variance \( G_\gamma^2 \), where \( G \) is the (co)variance matrix for the additive QTL effects, represented by the proportion of alleles IBD. All other terms are defined above. IBD coefficients between all individuals were determined using a deterministic algorithm (Pong-Wong et al. 2001) at 1 cM intervals along each chromosome. At each position the variance explained by the QTL effect was tested for significance using a standard LRT (LRT position the variance explained by the QTL effect was tested for 2001) at 1 cM intervals along each chromosome. At each

**Sample collections**

Ewes with a high probability of having the TT genotype, based on results from the segregation analyses described above, were selected, along with putative ++ controls, for detailed analyses. Estrous cycles were synchronized by withdrawal of intravaginal progestagen-impregnated sponges (60 mg medroxy-progesterone acetate per sponge; Intervet Laboratories Ltd, Cambridge, UK). On day 11 of the subsequent luteal phase luteolysis was induced by injection (i.m.) of prostaglandin \( F_2\alpha \) (PG; 100 mg Cloprostenol; Estrumate; Coopers Animal Health Ltd, Crewe, Cheshire, UK). Blood samples (3 ml) were taken daily by jugular venepuncture from day 6 to day 11 and then every 10 min via jugular catheter 24–30 h post-PG after which the animals were killed using an overdose of sodium pentobarbitone (Euthatal; Rhone-Merieux Ltd, Harlow, Essex, UK). It was not possible to carry out estrous cycle synchronization in some predicted TT ewes due to reproductive tract abnormalities. However, blood samples were taken from these noncyclic ewes at the same times as other animals. In order to assess cell proliferation within the ovary, ewes were injected with BrdU (Roche Diagnostics Ltd), administered ~2 h prior to death by two separate injections (total dose ~4.5 mg/kg) via jugular catheter, 10 min apart. At post-mortem the reproductive tracts were excised and examined. The ovaries were removed and halved, and either fixed in Bouin’s fixative for histological examination, or frozen for RNA/DNA preparation. Small pieces of spleen were also fixed for checking BrdU incorporation.

**GDF9 sequencing and mutation detection**

Total RNA was extracted from pieces of ovary using TRI Reagent (Sigma–Aldrich Ltd). First strand cDNA synthesis was performed using Omniscript RT Reagents (Qiagen Ltd). The entire GDF9 coding region was then amplified by PCR using Herculase II fusion DNA polymerase (Stratagene, Amsterdam, The Netherlands) and three sets of overlapping primer combinations designed from the published sheep sequence (AF078545; Bodenstein et al. 1999). The primers pairs used were as follows: G17: 5'-CACACGTAAGCTTAAAAGTGC-3' and G15: 5'-GATGCTGAGCTGTTTCTCG-3'; G10: 5'-CAACACTGTCGCTCCTACC-3' and G11: 5'-CAATTCAGGCTGGACACTCC-3'; G16: 5'-AATGATGGAGATTGATGTACG-3' and G7: 5'-TCTCTCCAAAGGCATAGAC-AGG-3'. The resulting PCR products were purified using a Wizard SV PCR Clean-Up System (Promega UK Ltd) and sequenced, using Big Dye 3.1 cycle sequencing chemistry, on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, CA, USA).

**Hormone assays**

Plasma LH and FSH were measured by RIA as previously described (McNeilly et al. 1976, 1986), using reagents kindly provided by Dr A Parlow (NIDDK, Torrance, CA, USA), with all samples being assayed in duplicate in the same assay. The limit of detection for NIH oLH-S18 was 0.3 ng/ml and for USDA-oFSH-SIAFP-RP2 (AFP 4117A) was 0.1 ng/ml. The intra- and inter-assay coefficients of variation (CV) were <10% for both assays. E2 was measured after extraction (Mann et al. 1995), using MAIA E2 kit (Serono Diagnostics). The limit of detection was 0.2 pg/ml and intra- and interassay CV were <12%. Progesterone was measured by RIA without extraction as described previously (McNeilly et al. 1992). The limit of detection was 0.2 pg/ml and intra- and interassay CV were <11%. Inhibin A was measured by ELISA as previously described (Knight et al. 1998, McNeilly et al. 2002). Assay sensitivity was ~30 pg/ml and intra- and interassay CV were <10%. Mean levels of plasma LH, FSH, progesterone, inhibin A and E2 in ++ and TT ewes were compared by unpaired Student’s t-test, performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

**Immunohistochemistry**

Tissues were immersed in Bouin’s fixative for 6 h, transferred to 70% ethanol, then dehydrated and embedded in paraffin wax. Sections (5 μm) were cut, floated onto Superfrost slides (BDH Laboratory Supplies, Lutterworth, Leics, UK), dried at 50°C for several hours then dewaxed and rehydrated. Some sections were stained with hematoxylin and eosin for morphological examination. Immunostaining was carried out for detection of...
BrdU, MVH, DAZL, ZP3, and laminin. Slides were washed in Tris-buffered saline (TBS; 0.05 M Tris pH 7.4, 0.85% NaCl) between treatments. For all antibodies antigen retrieval was carried out by pressure cooking for 5 min at full pressure in 0.01 M citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by incubating sections in 3% (v/v) hydrogen peroxide in methanol for 30 min. Non-specific binding of avidin/biotin system reagents was blocked by incubating in avidin for 15 min, then in biotin for 15 min (Avidin/Biotin blocking kit, Vector Laboratories, Peterborough, UK). Sections were blocked using 20% normal goat serum (BrdU, MVH, DAZL & laminin) or 20% normal rabbit serum (ZP3), 5% BSA in TBS for 1 h, then incubated overnight at 4°C in primary antibody diluted in blocking buffer as indicated. Primary antibodies and concentrations/dilutions were as follows: mouse anti-BrdU (1:170;376; Roche Diagnostics Ltd) at 2 μg/ml; rabbit anti-DDX4/MVH (ab13840; Abcam plc, Cambridge, UK) at 2 ng/ml; mouse anti-DDX4/MVH (ab17224-1; Abcam plc) at 1:200 dilution; rabbit anti-laminin (ab11575; Abcam plc) at 10 ng/ml; monkey anti-DDX4/MVH (ab13840; Abcam plc) at 10 ng/ml; monkey anti-ZP3 (bleed 723R; kindly gifted by Dr M Patterson, MRC, Edinburgh, UK) at 1:200 dilution; rabbit anti-laminin (ab11575; Abcam plc) at 10 ng/ml; monkey anti-ZP3 (bleed 723R; kindly gifted by Dr M Patterson, MRC, Edinburgh, UK) at 1:200 dilution. Antibody binding was visualized by incubation with rabbit anti-monkey peroxidase (Sigma–Aldrich Ltd) diluted 1:200, for 1 h. Antibody binding was visualized by incubation with streptavidin/HRP conjugate (Dako UK Ltd) diluted 1:200. Slides were then incubated with biotinylated goat anti-rabbit (BrdU, DAZL) or anti-mouse (MVH, laminin) IgG (Dako UK Ltd, Ely, Cambridgeshire, UK) diluted 1:500, for 30 min, then with streptavidin/HRP conjugate (Dako UK Ltd) diluted 1:1000, for 30 min. ZP3 slides were incubated directly with rabbit anti-monkey peroxidase (Sigma–Aldrich Ltd) diluted 1:200, for 1 h. Antibody binding was visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB+ substrate chromogen system; Dako UK Ltd). Sections were counterstained with hematoxylin, dehydrated, and then coverslips mounted using Pertex mounting medium (Cellpath, Newtown, Powys, UK). Images were photographed using an Olympus Provis microscope (Olympus Optical Co., London, UK) and a Canon EOS 30D camera (Canon UK Ltd, Reigate, Surrey, UK).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-09-0193.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was funded by the Medical Research Council (L Nicol, A S McNieely; U.1276.00.002.00007.01), the Rural Environment Research and Analysis Department of the Scottish Government (S M Rhind), and the BBSRC through the Roslin Institute ISPG (S C Bishop, R Pong-Wong).

Acknowledgements

The authors wish to acknowledge the staff of the Macaulay Institute research stations at Sourhope and Glensaugh and the University of Edinburgh staff at the Marshall Building, Roslin. We also thank Mr Ian Swanston and Mrs Nancy Evans for performing estradiol, inhibin A and progesterone assays and Dr Judy McNeiely for assistance with the gonadotropin assays.

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