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INVERDALE: A CASE STUDY IN GENE DISCOVERY

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SUMMARY
In July 2000 AgResearch announced the discovery of the genetic mutation responsible for the effects on reproduction seen in Inverdale sheep. The finding was the culmination of many years of research involving breeding and segregation studies, genetic linkage mapping, physiology, molecular biology and comparative links to studies in humans and mice (Galloway *et al*. 2000). This paper describes and reflects on key points of the finding which are relevant for future gene discovery.
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INVERDALE SHEEP
The Inverdale high fecundity gene ($FecX^I$) is a major gene for prolificacy in sheep which was first identified in descendants of a Romney ewe (A281) with consistently high litter sizes. Segregation studies showed that the gene is carried on the X-chromosome (Davis *et al*. 1991). A single copy of the gene in heterozygous (I+) ewes increases ovulation rate by about one extra egg, and litter size by about 0.6 lambs per ewe lambing. However, homozygous (II) ewes carrying two copies of the gene have small non-functional ovaries and are infertile (Davis *et al*. 1992).

GENE MAPPING
The ability to map the gene required several criteria: a clear phenotypic measurement of the gene effect, family pedigrees with the gene segregating, DNA markers and a genetic linkage map, and information about the physiology and biology likely to be involved.

The Inverdale phenotype is measured directly by laparoscopy in females and by progeny testing in males. The discovery that double-copy females are infertile greatly aided the selection of animals for genetic linkage mapping pedigrees, as this phenotype could be measured at 6 months and provided a certainty of carrier status. All full-sib fertile sisters of infertile ewes were also able to be phenotyped as I+ carriers with certainty without the need for ovulation rate measurments. Carrier rams could be progeny tested by mating to I+ carrier ewes and identifying at least one infertile daughter.

The Inverdale linkage mapping families consisted of 177 animals in a three-generation structure (with a maximum of 96 informative meioses) comprising known infertile (II) ewes and their sisters in the third generation (Galloway *et al*. 2000). A genetic linkage map of the sheep X-chromosome was constructed (Galloway *et al*. 1996), and the location of the Inverdale gene was progressively

narrowed down to a 10 cM region at the centre of the chromosome. This region of the sheep X-chromosome contained genes located around Xp11.2 –11.4 on the human X-chromosome.

Apart from an increase in ovulation rate carrier (I+) ewes appeared indistinguishable from their non-carrier counterparts, but infertile (II) ewes showed severe disruption of their normal ovarian function. The levels of circulating FSH and LH were as high as in ovariectomised ewes, and there was no detectable estradiol or inhibin (Shackell *et al.* 1993). Histological examination of the ovaries of adult ewes revealed no normal follicles developing beyond the primary stage (Braw-Tal *et al.* 1993) and similar arrest of follicle development was observed in samples of foetal ovaries (Smith *et al.* 1997).

LIKELY CANDIDATES

In addition to narrowing down the chromosomal location of the Inverdale gene by linkage mapping we considered several likely candidate genes which could contribute to the phenotype. The most notable of these was GDF9 (growth differentiation factor 9, a member of the transforming growth factor super-family (TGFβ)) which had been shown to be involved in fertility in mice. Mice with the GDF9 gene missing were infertile, and specifically blocked in follicle development beyond the primary stage (Dong *et al*. 1996). In humans and mice the GDF9 gene was not located on the X-chromosome, so it was unlikely to be Inverdale, but we verified this by mapping GDF9 in sheep to chromosome 5 (Sadighi *et al*. 1998).

However, the similarity between phenotypes of Inverdale sheep and GDF9 knockout mice alerted us to the likely possibility that, while not actually being GDF9 itself, the Inverdale gene may belong to a biochemical pathway involving GDF9 – perhaps Inverdale was a receptor for GDF9?

We then became aware of a new growth factor, GDF9B (also known as BMP15), closely related in sequence to GDF9. GDF9B was also produced by the developing egg (Laitinen *et al*. 1998; Dube *et al.* 1998; Aaltonen *et al.* 1999; Jaatinen *et al*. 1999), and the gene for GDF9B was located on the X-chromosome in humans and rodents, making it a very likely candidate for Inverdale. Our collaboration with the Finnish group who had discovered GDF9B in mice and humans led quickly to the sequencing of the gene in Inverdale sheep and the discovery of a point mutation in a region of the gene which was thought to be involved in biological activity of the protein molecule.

PROVING THE POINT

Many discoveries include a certain amount of serendipity, and for us that arrived in the form of a second flock of Romney sheep which appeared to be carrying the same gene as Inverdale sheep. Tokoroa farmer Mac Hanna had observed increased litter size and infertility within a line of his sheep and a small flock from this line was included in our studies (Hanna 1995). Crossing Inverdale and Hanna carriers produced infertile females indistinguishable from infertile (II) Inverdale females and thus implied that the same gene was involved. The increase in ovulation rate of Hanna carrier ewes was the same as that seen in Inverdale carrier ewes (Davis *et al.* 2001). Sequencing the GDF9B gene from Hanna sheep showed another mutation, different from the Inverdale mutation, which caused a premature stop signal in the gene. The practical result of this type of mutation would effectively mean that there was no functional GDF9B protein produced in these animals. This finding established that GDF9B mutations were the cause of the Hanna and Inverdale phenotypes, and indicated that the

small protein change in Inverdale GDF9B was equally as disruptive as the large protein deletion in Hanna sheep.

We mapped GDF9B onto the sheep X-chromosome in the same interval as the Inverdale gene and found no recombinants between the Inverdale phenotype and GDF9B in 78 co-informative meioses (LOD score of 23 for linkage between Inverdale and GDF9B). Expression of GDF9B could only be detected in sheep ovary samples and not in any other tissues (liver, heart, kidney, muscle, pituitary, uterus, placenta and adrenal). *In situ* hybridisation of a GDF9B detection probe to ovary tissue sections showed that GDF9B is only turned on within the eggs and first appears in primary follicles at about the same developmental phase at which the phenotype of arrested follicles is seen in infertile Inverdale and Hanna ovaries.

Comparison of the GDF9B protein sequence with other members of the TGFβ superfamily showed that the Inverdale mutation was not likely to have a major effect on overall shape and structure of the protein, but more likely to change the electrostatic surface charge of the protein and interfere with the protein surfaces needed for forming dimers. All TGFβ proteins are biologically active as dimers.

IMPLICATIONS

The discovery of the Inverdale gene has shown that GDF9B is essential for female fertility and that the effect it has on ovarian function is related to dose. The discovery has also highlighted a number of issues related to searching for genes.

First, the gene mapping and linkage studies were greatly aided by a clear and unambiguous phenotype (infertility as opposed to ovulation rate) and this is not always readily available for other traits being mapped. Second, a detailed understanding of the histology and developmental stage at which the effect occurred provided more data to enable close matching of phenotype to candidate genes. Third, the likely candidate gene was a small gene and easily sequenced, and the mutation was in the coding region of the gene. Fourth, the existence of a second family carrying a different and more obvious mutation in the same gene shifted the discovery from an association to a certainty that this gene was responsible. Without the Hanna line we would have had to rely on further physiology and cell biology to prove that the mutation was causing the Inverdale phenotype. Fifth, collaboration with a research group with intimate knowledge of the most likely candidate in other species speeded the discovery and brought another set of skills which strengthened the work. This has led to further collaborative work on the function of the Inverdale gene and provided a link to current discoveries on ovarian function in humans and mice. And sixth, discovery of the mutation in sheep has highlighted differences in the expression of GDF9B between rodents and larger mammals (humans and sheep), and has illustrated the way in which discoveries in livestock can have major implications in humans.

Use of the Inverdale gene in commercial flocks has shown that Inverdale ewes average 35% higher lambing than non-Inverdale ewes on the same property (Gray and Davis 1995). The Inverdale gene is gaining popularity within the industry in flocks where this substantial increase is desired. As it is carried on the X-chromosome then all daughters of a carrier ram inherit the gene, so no testing is required for these animals. However, the production of carrier rams from their female carrier parent does require a test. With the discovery of the specific mutations, the current Inverdale marker test

based on haplotype analysis of three markers around the gene (Galloway *et al.* 1999) has now been superceded. Previous testing required knowledge of the parents' haplotype in the region but animals can now be tested as carriers of the mutation without needing to test parents. We are currently developing an automated test for the mutation based on Mass Spectrometry.

GDF9B is a small secreted protein, and as such may be amenable to modification and useful in therapies for fertility control in a variety of species. Clearly much more work is needed to investigate the way in which GDF9B and GDF9 function to regulate follicle development, and in particular to determine how a smaller dose of GDF9B (in I+ carriers) causes increased ovulation rate.

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