The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene

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Abstract

Genetic variations in ovulation rate which occur in different breeds of sheep provide useful models to explore the mechanisms regulating the development of antral follicles. The Booroola gene, an autosomal mutation that affects ovulation rate, has been known for over two decades and despite intensive research it has not yet been identified. Using resources from human genome mapping and known data about gene linkage and chromosome location in the sheep, we selected the gene encoding the Bone Morphogenetic Protein receptor (BMPR) type 1 B (ALK-6) as a candidate site for the mutation. The BMPR1B gene in the human is located at the region linked with the Booroola mutation, syntenic to chromosome 6 in the sheep. A fragment of the sheep BMPR1B gene was cloned from an ovarian cDNA and the deduced aminoacid (AA) sequence is over 98% homologous to the known mammalian sequences. cDNA and genomic DNA from 20 Booroola genotypes were screened and two point mutation were found in the kinase domain of the receptor, one at base 746 of the coding region (A in the ++ to a G in FF animals) which results in a change from a glutamine in the wild type to a arginine in the Booroola animals. Another point mutation was identified at position 1113, (C to A) but this mutation does not change the coding aminoacid. The first mutation was confirmed in genomic DNA from 10 ewes from an independent Brazilian flock which segregates the Booroola phenotype. In all instances homozygous FecB gene carrier (n=11) had only the 746 A to G mutation, non gene carriers (n=14) had only the wild type sequence and heterozygote gene carriers (n=5) had both sequences. This mutation in the subdomain 3 of the kinase domain could result in an alteration in the expression and/or phosphorylation of SMADs, resulting in the phenotype characteristic of the Booroola animals which is the 'precocious' development of a large number of small antral follicles resulting in increased ovulation rate.

Journal of Endocrinology (2001) **169,** R1–R6

Introduction

The careful regulation of the number of eggs shed and hence the litter size is crucial to successful reproduction in all species (Baird & Campbell 1998). It would be expected therefore that the mechanisms involve genes which are conserved throughout evolution. The ovulation rate (OR) in sheep of different breeds range from one to as many as 10 in the Booroola Merino, providing a convenient model to study the molecular genetics of this phenomenon.

The Booroola gene is an autosomal mutation identified on the basis of segregational studies on litter size (Piper & Bindon 1982) and OR (Davis *et al.* 1982). This phenotype has a Mendelian pattern of segregation indicating that it is caused by a major gene, which has additive effects on OR and is dominant for litter size. The alleles were called FecB (F) for the putative high prolificacy allele and Fec+ (+) for the wild type. Based on the segregation of the OR in Merino and Romney flocks, the genotypes in the ewes have been classified as homozygous non-carrier (++) with an OR of 2 or less, heterozygous carriers (F+) with OR of 3–4 and homozygous carriers (FF) with more than 5 ovulations per cycle (Davis *et al.* 1982). To date there is no characteristic phenotype for the different genotypes in males, so their genotype is classified based on their pedigree and progeny (Hochereau-de Reviers *et al.* 1997).

In sexually mature ewes the most obvious effect of the Booroola mutation is seen in the ovaries. The total population of antral follicles and the proportion of non-atretic follicles is similar among genotypes, but the ovulatory follicles are smaller and have fewer granulosa cells in ewes carrying the Booroola mutation (McNatty *et al.* 1985,

Journal of Endocrinology (2001) **169,** R1–R6 Accepted 21 March 2001
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Driancourt *et al.* 1985, McNatty *et al.* 1986, Souza *et al.* 1994). Despite the differences in follicle number, the total number of granulosa cells from oestrogenic follicles per animal are similar among genotypes (McNatty *et al.* 1986).

The members of the transforming growth factor beta (TGF-b) superfamily of peptides which includes the bone morphogenetic proteins (BMPs) and activins are involved in the regulation of the development and homeostasis of most tissues in several animal species ranging from insects (fruit fly) to mammals (Raftery & Sutherland 1999). The TGF-b signal transduction network involves receptor serine/threonine kinases at the cell surface which after binding of a ligand to its type 2 receptor in concert with a type 1 receptor leads to a formation of heterodimeric receptor complex and phosphorylation of the type 1 receptor. Once activated, the type 1 receptor phosphorylates a receptor-regulated Smad which then heterodimerizes with a common Smad (Smad-4) and moves to the nucleus where the Smad complex associates with nuclear transcription factors and activates transcription of target genes (Massague 1998).

TGF b signalling is essential in the gametogenesis of mammals. Mouse BMP4 homozygous null embryos contain no primordial germ cells (PGCs) while heterozygotes have fewer PGCs than normal (Lawson *et al.* 1999). Expression of BMP 4 and 7 has been identified in the theca cells and BMP receptor (BMPR) types 1A, 1B, and 2 in the granulosa cells and oocytes of most follicles in ovaries of normal cycling rats. Physiological concentrations of BMP 4 and 7 induced rat granulosa cytodifferentiation enhanced FSH induced oestradiol synthesis and attenuated progesterone production.(Shimasaki *et al.* 1999).

The gene mutation responsible for the Booroola phenotype has not yet been identified but analysis of segregation identified linkage markers from human chromosome 4 at a region between 4q21–25 (Montgomery *et al.* 1993). Additionally, it has been mapped to sheep chromosome 6 in a region between SPP1 and EGF (Montgomery *et al.* 1995). From over 50 genes coding for ligand, receptor, and intracellular signalling peptides of the TGF-b superfamily, we have selected BMPR1B after a report of the cDNA cloning of human receptor and the localisation to chromosome 4 at the region between 4q22–24 (Astrom *et al.* 1999), which corresponds to the expected location of the Booroola mutation. Hence a mutation in the BMP signalling system could have physiological importance in triggering the phenotype consistently observed in the Booroola animals which is the development of a large number of follicles that mature at a smaller size with fewer granulosa cells than wild type animals.

Materials and Methods

Cloning of sheep BMPR1B

DNA fragments of the sheep BMPR1B genes were isolated by polymerase chain reaction (PCR) using primers based on homologous regions of the sequence of human and mouse BMPR1B genes (primer 1: 5′-CATTCC TCATCAAAGAAGAT-3′, primer 2: 5′-AGTGTTCTT CACCACAGAG-3′, primer 3: 5′TGATTATCTGAA GTCCACCACC3′ and primer 4: 5′CAAAATGTCAGA GTCCCAGGACAT3′). RNA was extracted using Rnazol B (AMS Biotechnology, UK) according to manufacturers instructions, from prepubertal sheep ovaries (with no antral follicles visible in the surface), reverse transcribed using the Advantage RT-for-PCR kit (Clontech, UK) and amplified using Taq DNA polymerase (Roche, UK) in a PTC-200 thermal cycler (MJ Research, UK). The PCR conditions were 49 °C annealing temperature for 35 cycles for fragment 1 (primers 1 and 2) and 60 °C annealing temperature for 35 cycle for fragment 2 (primer 3 and 4). Products of the expected size (457 and 635 base pairs [bp]) were obtained, the DNA fragments were cloned into pGEM-T Easy (Promega, UK) and verified by sequencing using an ABI 373XL sequencer. Subsequently a third pair of primers was used to produce a sequence product which would overlap of the sequences from the two fragments (primer 5: 5′TGGATGGGAAAGTG GCGTGG3′ and primer 6: 5′GTGAAGAAAAATGG AACTTGCTG3′).

Screening for mutations in Booroola genotypes

Ovarian cDNA from 5 homozygous FF and 5 homozygous ++ Scottish Blackface Merino cross ewes, in which the genotype was determined by pedigree (Haley 1990) were used as a template for PCR reaction using PWO DNA polymerase (Roche, UK). Two fragments of around 800 bp were amplified, the first coding for the extracellular domain of the receptor up to the transmembrane domain (primer 1 and 6, 49 °C annealing temperature), and the second fragment comprised the intracellular domain of the receptor (primer 4 and 5, 60 °C annealing temperature). The amplified PCR fragments were gel purified (Agarose gel DNA extraction kit, Roche, UK) and directly sequenced. The obtained sequences were compared between the genotypes using the GeneJockey II software (Biosoft, UK).

Genomic DNA studies

To confirm the previous observations using ovarian cDNA preparations, genomic DNA was obtained from a further 5 homozygous FF and 5 homozygous ++ ewes from the same flock in which the genotype was determined by measurements of the OR by laparoscopy on at least 3 occasions (Haley 1990). Jugular blood samples were collected and the genomic DNA was extracted using Puregene DNA extraction Kit according to manufacturer instructions (Flowgen, UK). PCR was performed with

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high fidelity enzyme using primers 5 and 6 at an annealing temperature of 57 °C for 35 cycles. The DNA product obtained was 1·3 kb, instead of the 386 bp predicted from the cDNA sequence, which indicated the presence of introns in the amplified fragment. The PCR product was sequenced and an antisense primer was designed close to the intron boundary (primer 7: 5′CTCATCTGTCTA GATCCGCTA3′). A new primer combination 5 and 7 was then used to amplify the fragment of interest from genomic DNA and verify the presence of the mutation in both DNA strands by sequencing.

The Booroola lines utilised to monitor for these mutations were all descendent from 2 ram heterozygote carriers of the FecB gene (C S Haley 1990). To exclude the possibility of a founder effect in the occurrence of this point mutation, genomic DNA from 4 wild type (++), 5 heterozygote and 1 FF ewe derived from another independent Brazilian flock (Moraes *et al.* 1991) were used to screen for this mutation.

Results

A partial sequence of the sheep BMPR1B gene was obtained (Figure 1A, GenBank accession AF357007) and the deduced aminoacid (AA) sequence of the sheep gene is 98·6%, 98·4%, 98%, 92·2% and 80·5% homologous to the predicted sequence of the mouse, human, rat, chicken and zebra-fish, respectively. The homology in the kinase domain (Figure 1A and B) is over 98% between sheep and other mammals, around 97% identical when compared with avian and 92% homologous with fish sequences.

The screening of ovarian cDNA from 10 ewes homozygous for the Booroola genotypes resulted in the identification of a single point mutation in the subdomain 3 (Hanks & Hunter 1995) of the kinase domain of the BMPR1B at base 746 of the coding region (A in the ++ to a G in FF animals, Figure 1C). This mutation results in a change in the amino acid coded from a glutamine in the wild type to an arginine in the Booroola animals (Figure 1A). Another point mutation (C in the ++ to a A in FF ewes) was identified at position 1113. However this mutation does not change the coding aminoacid, but does result in a loss of a Xho I restriction site in the ++ DNA and the gain of an BstU I restriction site in the FF ewes.

The PCR product amplified from genomic DNA primers 5 and 6, resulted from the presence of a single intron which span for 890 bp and starts at the base 777 of the coding region. The 746 mutation was confirmed (primers 5 and 7 PCR product) in genomic DNA from a further 10 ewes from the local flock and 10 ewes from an independent Brazilian flock which segregates the Booroola phenotype. In all instances homozygous FF animals (n=11) had only the 746 A to G mutation, non gene carriers ++ ewes (n=14) had only the wild type sequence and heterozygote F+ animals (n=5) had both sequences (Figure 1C).

Discussion

These results clearly indicate a very strong association with the Booroola phenotype segregation and the mutation described in the BMPR1B gene. The glutamine 249 which is mutated in the Booroola (glutamine in ++ to a arginine in FF) is conserved in all vertebrate species from which the sequence is published and is also conserved in the orthologous Decapentaplegic (Dpp) type 1 receptor Thickvein (Tkv) in the fruit fly. The association of this mutation was consistent in the DNA from over 25 ewes tested with distinct Booroola phenotypes. Further, its presence in a flock from a totally independent origin and incorporated into different breeds, excludes the possibility of a founder effect for this mutation.

A mutation in the kinase domain of the BMPR1B is likely to have significant implications in the signalling of this receptor and since BMPs have been implicated in several aspects of gametogenesis, this mutation may be consistent with the phenotype observed in Booroola ewes. Because the predicted AA substitution it is located close to the L45 loop, which is critical in determining specific SMAD isoform activation (Persson *et al.* 1998, Chen & Massague 1999), this mutation could lead to alterations in the expression of SMADs and/or phosphorylation patterns, resulting in the phenotype characteristic of the Booroola mutation.

The BMP 2 ortholog Dpp is essential for Drosophila oogenesis and is required for patterning of anterior eggshell, similar requirements are also revealed by mutations in the Dpp receptor type 1 Sax. A loss of Sax function in the germline results in a block in oogenesis associated with egg chamber degeneration and a failure of the transfer of nurse cell contents to the oocyte, indicating that TGF-beta signalling is required for these events (Twombly *et al.* 1996). Adult female flies mutant for the myogenic myocyte enhancer binding factor-2 (MEF2) are weakly fertile and produce defective eggs, this mutation results in overexpression of Tkv receptor RNA in the egg chambers and abnormal patterning and differentiation of the follicle cells (Mantrova *et al.* 1999). The manipulation of Dpp levels also leads to alteration in the follicular epithelial cell pattern of differentiation in the anterior eggshell structures resulting in the formation of enlarged operculum (Dobens & Raftery 1998). Thus the BMP equivalent system in Drosophila is very important in oogenesis.

BMP signalling is also essential for folliculogenesis in mammals. Mouse BMP 4 knockout embryos contain no primordial germ cells (PGCs) and also lack other extra-embryonic mesoderm derived tissue such as the allantois, which along with the PGCs originate from the proximal epiblast. BMP 4 heterozygotes have fewer PGCs than

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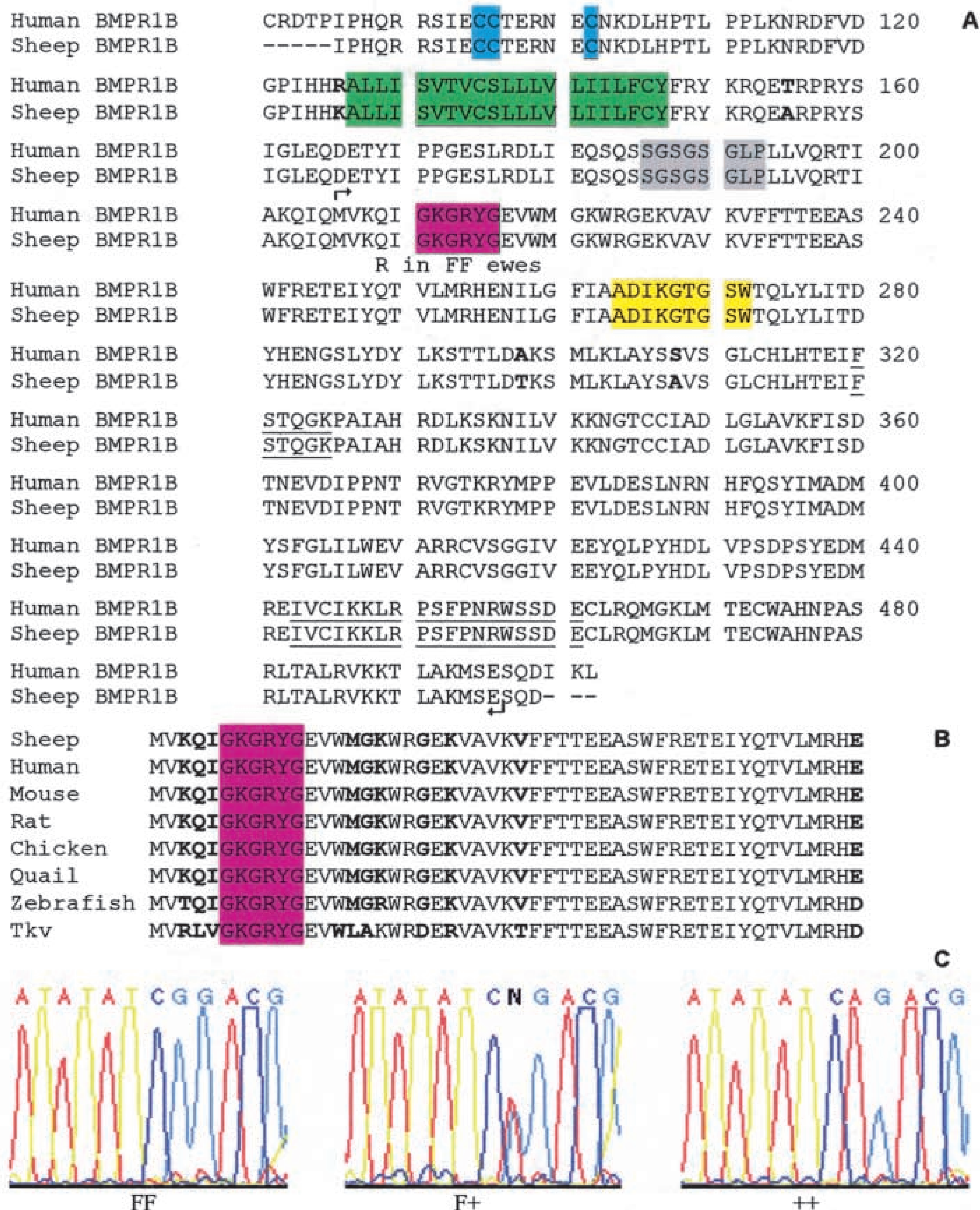

Figure 1 A: Comparison of protein sequence of the BMPR1B from human and sheep, important domains of the receptor are indicated by the following colour scheme. Cysteine residues in the extracellular domain (cyam), transmembrane domain (green), GS motif (grey), arrows indicate the border of the kinase domain, ATP binding site (magenta), loop 45 (yellow), kinase inserts (underlined), areas of non homology are indicated in bold letters. B: Comparison of the kinase domain of the species which the BMPR1B gene sequence is known and the Drosophila ortholog Tkv. Figure 1C: Example of the sequence obtained for each of the genotypes illustrating the presence of both sequences in the heterozygotes (F+). The GenBank accession numbers of the BMPR1B genes were: human (NM001203), sheep (AF357007), mouse (Z23143), rat (JC2491), chicken (Q05438), quail (AF189778), zebrafish (AB020758) and the Drosophila ortolog Tkv (U11442).

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normal, due to a reduction in the size of the founding population (Lawson, *et al.* 1999). Strong mRNA expression for BMP 4 and 7 has been identified in the theca cells and BMP receptor (BMPR) types 1A, 1B, and 2 in the granulosa cells and oocytes of most follicles in ovaries of normal cycling rats. Physiological concentrations of BMP 4 and 7 induced rat granulosa cytodifferentiation and enhanced FSH induced oestradiol synthesis and attenuated progesterone production. Similarly the BMP receptor types 1A, 1B, and 2 are expressed in the granulosa layer of sheep follicles throughout folliculogenesis and treatment *in vitro* of immature granulosa cells with recombinant BMP2 results in increased oestradiol production due to early differentiation of the cells (Souza *et al.* 2000). Moreover, a deletion in the BMP 15 gene (GDF-9b) has been recently shown to cause the Inverdale phenotype in ewes, which result in increased OR in the heterozygotes and disrupted follicle development of the homozygous carriers (Galloway *et al.* 2000). Furthermore, crosses between Inverdale and Booroola genotypes have an additive effect on OR, since when the 2 genes were in combination the OR was higher than the sum of the effects of each gene alone (Davis *et al.* 1999) suggesting that both phenotypes share a common pathway of signalling.

The BMP have also been implicated in development of several organs such as heart, brain and adrenal which is consistent of the action of the Booroola mutation during fetal life. We have recently observed that the weight of the adrenal glands in FF ewes is significant less than in wild type animals (Souza & Baird, unpublished results).

The FecB gene in Booroola fetuses from day 28 of gestation, is associated with retarded development of the heart (day 28), mesonephros (days 30–40) and in the ovary from day 30 to early neonatal life (McNatty *et al.* 1995). The ovary of females carrying the FecB gene, have fewer oogonia (days 30–40), primordial follicles (day 75–90) and growing follicles (day 120 to 6 weeks after birth). By contrast, the Booroola gene is not associated with differences in plasma gonadotrophin or immunoreactive inhibin until early neonatal life (McNatty *et al.* 1995).

A mutation like the Booroola that causes profound disruption in the mechanism controlling OR is likely to be lethal for essentially monovulatory species like the human and cow due to excess numbers of fetuses implanting in the uterus which results in major fetal loss or death of the mother. It is detrimental when it is homozygous in species with intermediate OR like the sheep, even when special care is provided during lambing (Villaroel *et al.* 1990, Amer *et al.* 1999). However, it is likely to be conserved in polyovulatory species like the pig and mouse, which normally cope with a large litter size. The maintenance of a mutation like this in the BMPR1B at least in the pig is suggested by reports of quantitative trait locus (QTL) increasing OR at syntenic region in the pig genome, in the chromosome 8 (Rathje *et al.* 1997, Rohrer *et al.* 1999, Wilkie *et al.* 1999)

Note Added in Proof

Since the submission of this paper the same mutation has been reported in a separate flock of Booroola ewes (Wilson *et al.* 2001. Biology of Reproduction **64** 1225–1235).

Acknowledgements

This work was supported by JRF Ltd Research Fellowship 2000–2/3 and MRC program grant G8929853. CMacD is funded by an MRC grant G9827407 (awarded to E Telfer, B Campbell & D Baird). We are grateful to Dr J C Ferrugem Moraes and Dr M Benavides for helpful discussion and for kindly providing the DNA from the Brazilian Booroola flock, to Ms J Docherty and Ms M Thomson for animal care and to Ms L Nicol and Ms G Crawford for help with the probes and sequencing.

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