Pakistan J. Zool., vol. 45(5), pp. 1351-1357, 2013

Identification of Molecular Markers in Bone Morphogenetic Protien15 (BMP15) Gene of Balochi Sheep

Ahmad Nawaz Khosa,[1] Masroor Ellahi Babar,[2] Asif Nadeem,[3] Tanveer Hussain,[3] Farah Bilal,[3]* Khalid Javed[2] and Khushi Muhammad[4]

[1]*Faculty of Veterinary and Animal Sciences, Lasbela University of Agricuture, Water and Marine Sciences, Uthal, Balochistan, Pakistan*

[2]*Deparment of Livestock Production, University of Veterinary and Animal Sciences, Lahore*

[3]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore*

[4]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore*

Abstract.- Balochi sheep is more prolific than other breeds of sheep found in Pakistan. Fertility rate in Balochi is 70-75%. A study was carried out on BMP15 gene of Balochi sheep. Sequencing data analysis revealed an insertion of three nucleotides CTT at nucleotide position 171, 172,173, and a deletion of three nucleotides TGA at nucleotides position 3477, 3478, and 3479 of BMP15 gene. Two polymorphism in nucleotide positions 3296 C>T and 4851 T>G were also identified. This study could provide basic molecular data on the reproductive characteristics and a scientific basis for the conservation and utilization of sheep breeds.

Key Words: Bone morphogenetic protein 15, BMP15, polymorphisim, nucleotide aligment.

INTRODUCTION

Sheep provide good scope for enhancing the supply of meat as well as animal protein. Pakistan is rich in sheep genetic resources. Pakistan has well recognized 28 sheep breeds. The purpose of small ruminant raisings is to produce quality meat to fulfill the demand of meat consumption. At present, there are 28.8 million sheep in Pakistan (Economic Survey of Pakistan, 2012-13).

The importance of small ruminants in general and high prolific animals in particular, is greatly increased in Pakistan due to ever increase in the population during the last decade. It is an established fact that an animal producing twins or triplet contributes more than 1.5 times toward meat than the animals producing single offspring per lambing. Among the some sheep breeds, the twinning percentage in fat tailed sheep breeds (Balochi) had the fertility rate as 70-75 percent (Rafiq and Munir, 1983). Due to low heritability of litter size, attempts to increase litter size by selection within a breed results in slow progress (Morris, 1990). Genetics of prolificacy in sheep emphasize the importance of main genes which have been made known to affect litter size and rate of ovulation through various mechanisms. Therefore, the identification of major genes which have great effects on ovulation rate and litter size has generated substantial interest among sheep breeders and scientists. Booroola is reported as the first major gene to enhance ovulation rate (Pennetier *et al.,* 2004; Eckery *et al.,* 2002). The two genes GDF-9 and BMP-15 have made their sources in oocytes (Juengel *et al.,* 2002). The BMPR-1B receptor of BMP is expressed by granulosa cells and the oocytes from the early to the late antral follicle stages and to a slighter amount by theca layer of ovine and bovine antral follicles (Souza *et al.,* 2002; Wilson *et al.,* 2001; Glister *et al.,* 2004).

The function of gene bone morphogenetic protein 15 (BMP-15) is not absolutely appreciated or understood even if gene collaborate to regulate granulose cells function (McNatty *et al.,* 2005). Earlier the present report have been described that at least five mutations in this gene affect the prolificacy. BMP15 gene has a vital role and necessary for folliculogenesis in sheep. If the same gene carrying two copies of naturally occurring inactivating BMP15 mutations are infertile and the follicular development will be blocked at the primary stage. In sheep it is furthermore clear that if heterozygotes mean carrying inactivating mutation

* Correspondance author: ankhoso2004@yahoo.com

0030-9923/2013/0005-1351 $ 8.00/0

in only one copy of BMP15 gene, whereby the other copy of the gene produces active protein, this situation likely increased ovulation rate (Galloway *et al.*, 2000). BMP15 gene is an X-linked gene in nature and it had made so easy to identify high prolific animals at an early stage of life because of the identification of FecB (Booroola) marker on sheep X chromosome. Markers that appreciably contribute to the variance of trait expression in livestock have been increasingly a focus in the field of livestock genetics. If such markers can be identified in Pakistani sheep breeds, identification and planned breeding of high prolific animals will result in fast vertical expansion of small ruminants and hence mutton prduction. There is comparatively less scientific knowledge available on small ruminants in this regard, and not a single study is done in Pakistan. Therefore, the present study deals with the fecundity gene Bone Morphogenetic Protein 15 (BMP15) in Balochi sheep.

MATERIALS AND METHODS

Animal selection and DNA extraction

To study the genetics of fecundity in sheep, fifty individuals of Balochi sheep with different families having no blood relation were examined. The blood samples of Balochi sheep were obtained from different Government Livestock farms (Karakul Sheep Breeding Farm Maslakh Quetta and Bhagnari cattle/Balochi sheep Farm Usta Mohammad District Jaffarabad Balochistan). Sheep having single and multiple births history were selected for blood collection. These animals were selected randomly avoiding the relationship equivalency at maximum. Fifty samples of Balochi sheep were sequenced for the present investigation. This type of selection of animals provides a unique research material to identify molecular markers associated with fertility. Ten (10) ml blood sample was collected aseptically from jugular vein of selected sheep in the tubes containing anticoagulants *i.e.* Ethylene diamine tetra-acetic acid (0.5 M EDTA). Field samples were placed in ice immediately after their collection and brought to the laboratory and stored temporarily in freezer at -20°C before DNA extraction. DNA was extracted by inorganic method (Sambrook and Russel, 2001). Quantity and quality of the DNA samples was measured.

Primer designing, optimization and polymerase chain reaction

Specific primers for the full length amplification of BMP15 gene were designed using software Primer3 (www. *http://bioinfo.ut.ee/primer3-0.4.0/).* Sequence and product size of the primers are given in Table I. Genome browser web facility of the already reported sequence of these genes available at NCBI was used. Primers were optimized for their annealing temperature. Primers were amplified by a temperature gradient PCR in which a range of annealing temperature (64°C to 54°C) was used in Bio-Rad thermo cycler. The temperature at which primer showed best results were observed and selected. The subsequent PCR were carried out at optimized annealing temperatures. Polymerase chain reactions were carried out in a 25 µL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 2 µM of each primer, 50 ng of ovine genomic DNA, and 1U of *Taq* DNA polymerase. The amplification conditions for primers of the BMP15 gene were as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 sec; annealing at 57°C for 30 sec; and extension at 72°C for 30 sec; with a final extension at 72°C for 10 min, on a BioRad Thermocycler. PCR products were purified by FavorPrep PCR purification mini kit for sequencing**.** Standard method was followed as described by manufacturer.

DNA sequencing and analysis

PCR products were sequenced in both directions using dideoxy chain termination direct Sanger sequencing on ABI genetic analyzer 3130. The sequence was blast against normal sequence by using Bioedit software version (Hall *et al.*, 1999). Sequences were aligned with and change in the DNA sequence was confirmed by sequencing both sense and antisense strands.

Analysis of sequencing samples

The sequence was also blast against normal sequence by using Bioedit software version

Table I.- List of Primers of BMP 15 Gene

Serial #	Direction	Primer	Length	Products Size (bp)
1	Fec9bGSF1	CTGCCAGCCTTTCATTTTTC	20	420
	Fec9bGSR1	TTTTCCCTAGGGTGTCCACTT	21	
2	Fec9bGSF2	CTGCCAGCCTTTCATTTTTC	20	274
	Fec9bGSR2	TGCCCTACCTGTGTCATTTG	20	
3	Fec9bGSF3	GATTCAGGAGCTGCTAGAAGAA	22	420
	Fec9bGSR3	TGAAGCCTGACAGAAAACTGA	21	
4	Fec9bGSF4	AGGCCGCTGGCTAGTGTAG	19	420
	Fec9bGSR4	AACAGCCCTCCACAGAACAT	20	
5	Fec9bGSF5	GGCAACTCATTGATGTGTCAG	21	420
	Fec9bGSR5	AAGCTCAACTCCTGCCTCTG	20	
6	Fec9bGSF6	CTGCTTAGCTGTCCTGAAAGG	21	420
	Fec9bGSR6	TTCCCTAGAGAACCCTGCAC	20	
7	Fec9bGSF7	GACTATGTTGCTAGTTTGGGTTTG	24	420
	Fec9bGSR7	TTGGGGAGGATTAAGGAAGA	20	
8	Fec9bGSF8	CCTCCCTCCTCCAAGTAAAA	20	420
	Fec9bGSR8	CTGAGCTTCATTTTCTTCACCT	22	
9	Fec9bGSF9	CAGGGATCAAACCCACATCT	20	420
	Fec9bGSR9	AGCCCCAGGAATCCTACTGT	20	
10	Fec9bGSF10	TTGGGGTTGGGTATAAAAAGG	21	420
	Fec9bGSR10	TCACATTCCAACACCCAGAA	20	
11	Fec9bGSF11	TGGATACAGGGAGGGAAGTG	20	420
	Fec9bGSR11	TTTCCACCTTTAGGCCTTTG	20	
12	Fec9bGSF12	GAGGTGACATTTGAGCTGAGG	21	420
	Fec9bGSR12	CCATTTCTGGATCCTTTCCA	20	
13	Fec9bGSF13	TCATAAGCTGCATAAGTCAATTCT	24	420
	Fec9bGSR13	ACCCTGCCTGAAAAGGAA	18	
14	Fec9bGSF14	CACCCTGCTTCAGGAAATATG	21	420
	Fec9bGSR14	GACCCGCTGGTAAACACTAATC	22	
15	Fec9bGSF15	AAGCATGAGTTGGAACCTGAA	21	420
	Fec9bGSR15	TCTCCACTGAATCCATGAGC	20	
16	Fec9bGSF16	TTTTGCACCTGAAACTTGGA	20	420
	Fec9bGSR16	TTGATACTTCTCCCGGCAAT	20	
17	Fec9bGSF17	GCTGTATATTGTCACCCTGCTT	22	420
	Fec9bGSR17	CTTTCCTTCCAAGGAGCAAG	20	
18	Fec9bGSF18	CATCTGGTCCCATCACTTCA	20	474
	Fec9bGSR18	GACTGGTTGGATCTCCTTGC	20	
19	Fec9bGSF19	TCAAGGCTATGGTTTTTCCAG	21	420
	Fec9bGSR19	TTCTGGAGTTCACCCAAACC	20	
20	Fec9bGSF20	CCACCTGATGTGAAGAGCTG	20	420
	Fec9bGSR20	AAAGCGTTGAAAAGCAGGAC	20	
21	Fec9bGSF21	TGAACTGAACTGATGGTTAGTGA	23	377
	Fec9bGSR21	TTGAAAGTGAAAGTCGCTCA	20	
22	Fec9bGSF22	GTTGGTAAGATCCCTGGAGAAG	22	420
	Fec9bGSR22	GGTTGGAGATGCCACAAAAT	20	
23	Fec9bGSF23	GGAACGGAAAAGAGGGAGAT	20	420
	Fec9bGSR23	CCCCTAGACGGAGAAAAACA	20	
24	Fec9bGSF24	TTCATATGTTTCAATGACCCTCTT	24	376
	Fec9bGSR24	GCTCTTGAATCCACAATAGCC	21	
25	Fec9bGSF25	AAACACTGGCTTGTGTGTCCT	21	420
	Fec9bGSR25	TATGCTACCCGGTTTGGTCT	20	
26	Fec9bGSF26	GCTTTGCTCTTGTTCCCTCT	20	420
	Fec9bGSR26	TGCCACCAGAACTCAAGAAC	20	
27	Fec9bGSF27	CCCAAAACTTGGACAGAGATG	21	420
	Fec9bGSR27	ATGCAATACTGCCTGCTTGA	20	
28	Fec9bGSF28	ACTCAGAGTGTTCAGAAGACCAAA	24	488
	Fec9bGSR28	CTGGGCAATCATACCCTCAT	20	
29	Fec9bGSF29	AGTGTTCCCTCCACCCTTT	19	348
	Fec9bGSR29	GCCTCAATCAGAAGGATGCTA	21	

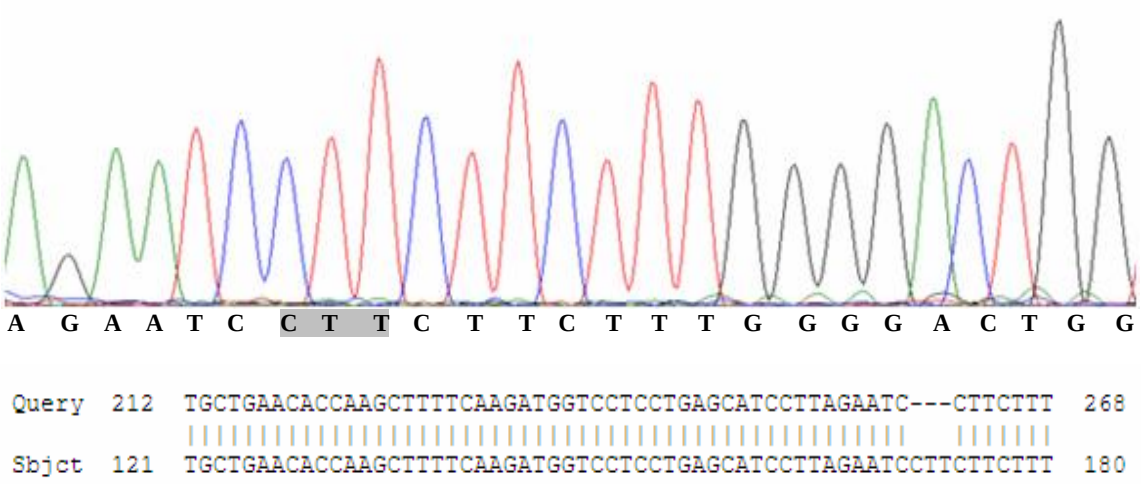

Fig. 1. Electropherogram of the identified insertion in Balochi sheep at nt position 171, 172, 173(CTT)

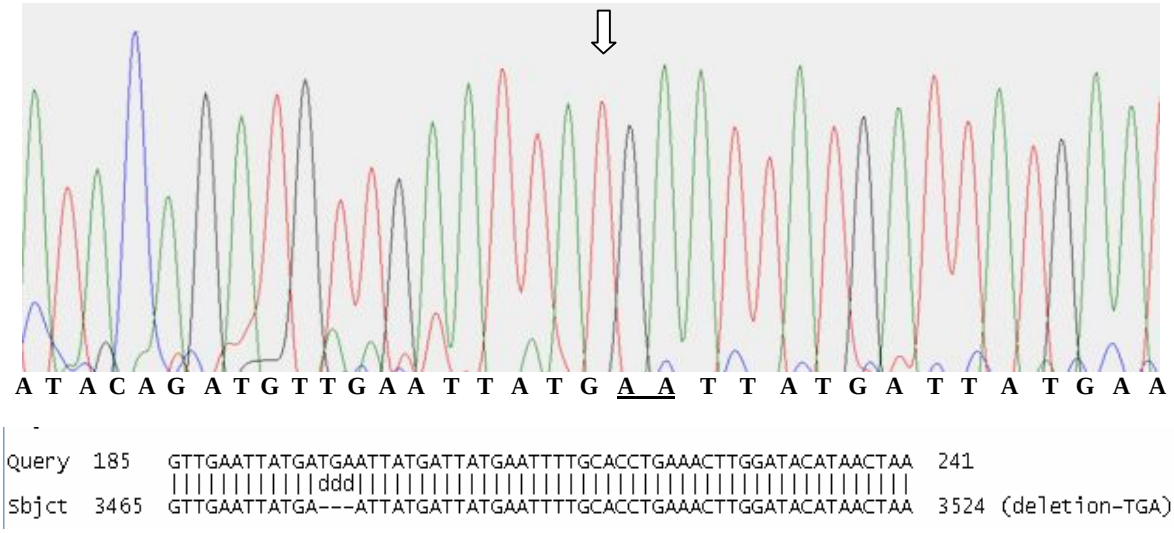

Fig: 2. Electropherogram of the identified deletion in Balochi sheep at position number 3477, 3478, 3479 (TGA)

Table II.- Identified polymophism in Blaochi sheep.

Sr. No	SNP ID	SNPs Position	Ref. Nucleotide	Nucleotide Change	Exonic/Intronic	Transition/ Transversion
1	BMS1	Ins(171,172,173)	-	CTT	Exonic	-
2	BMS2	3296	C	T	Intronic	Transition
3	BMS3	3477	T	Del	Intronic	-
4	BMS4	3478	G	Del	Intronic	-
5	BMS5	3479	A	Del	Intronic	-
6	BMS6	4851	T	G	Intronic	Transversion

(Hall *et al.*, 1999). Sheep sequences were aligned with accessation # AF236078 for exon 1 and AF236079 for exon 2. Any change in the DNA sequence was confirmed by sequencing both sense and antisense strands. Analysis of the sequences was done with the help of appropriate softwares.

RESULTS

Identified polymorphic sites in Balochi sheep breeds are shown in Table II. Sequencing data analyses revealed that in the exonic region an insertion of three nucleotides CTT at nucleotide position 171, 172, 173, and a deletion of three nucleotides TGA at nucleotides position 3477, 3478, and 3479 of BMP15 gene were observed in the intron, a transition nucleotide positions 3296 C>T and a transversion 4851 T>G were also observed in the intron.

Statistical analysis of identical polymorphism in Balochi sheep shows an insertion of three nucleotide (CTT) at position 171,172,173 had P-value 2.40E-04 and deletion of three nucleotides (TGA) at nucleotide position 3477, 3478, and 3479 have the P-value 4.47E-03, 4.47E-03, 4.47E-03, respectively (Table III). P-value of 3296 and 4851 polymorphic sites were observed as 5.16E-02 and 8.03E-02.

Codon modification of BMP15 gene in Balochi sheep is presented in Table IV. DNA Sequencing analysis of BMP15 gene revealed an insertion polymorphism of leucine (CTT) in Balochi sheep at codon 10.

Table III.- Statistical analysis of the identical polymorphism in Balochi sheep.

S. No.	SNP ID	Chromosomal position	P-Value
1	BMS1	Ins(171,172,173)	2.40E-04
2	BMS2	3296	5.16E-02
3	BMS3	del 3477	4.47E-03
4	BMS4	del 3478	4.47E-03
5	BMS5	del 3479	4.47E-03
6	BMS6	4851	8.03E-02

Table IV- Codon modifications of BMP15 gene in Balochi sheep.

SNPs position #	171, 72, 73
Codon #	100
Reference sequence	CTT
Changed sequence	Insertion
Reference amino acid	-
Changed amino acid	Leucine

DISCUSSION

In the earlier period, escalating the number of lambs born in the flock was mostly inadequate due to the use of the breeding program. No doubt, selection can play an important role but it is significantly slower process and may take decades to make almost 20% improvements in prolificacy. Despite this, nowadays molecular markers offers a new option that can allow the commercial producer to create a high level of prolificacy and still retain the important other traits such as high prolificacy, lamb growth rate, carcass and heavy quality fleece are all desired in the flock.

The genetics of sheep litter size has been well documented with a number of imperative prolificacy genes of sheep. Various studies on the genetics of prolificacy in sheep emphasize the importance of main genes namely BMP15, BMPR1B and GDF9. These three important genes which have been known to affect litter size and rate of ovulation through various mechanisms (Davis, 2005). BMP15 (Bone Morphogenetic Protein 15) is considered the important gene having the potential role in the fecundity of the sheep. BMP15 gene, also called, GDF9b, has been mapped to the sheep X-chromosome comprising 6648 nucleotide full length coding sequence and contains two exons which are separated by an intron of about 5309 nucleotides and encodes protein of 394 amino acid (Galloway, *et al.,* 2000).

In this study, fine mapping of BMP15 genes was performed to identify the polymorphism and their association with litter size. Balochi sheep breed of Pakistan was selected to see the nucleotide picture of this gene. It is well reported that BMP15 gene has significant role in fecundity so a number of polymorphism, insertion and deletion in Balochi sheep breed was identified and correlated with litter size of the sheep. A lot of work has been reported on sheep BMP15 gene but polymorphism is not abundant. In the present research, various polymorphisms, insertion and deletion in Balochi sheep breeds were identified and associated with fecundity and secondly, some novel polymorphisms were identified which are different from the sheep breeds of the world. These novel polymorphism will

be helpful to identify the region of the sheep breeds of the world. Sequences of same gene from Balochi sheep breed has also been submitted to the NCBI GenBank and given the accession # JN655672. Selected sheep breed for the research are very well known breed of Pakistan. The Balochi sheep is highly fertile with almost 70-75% fertility. In balochi sheep breed, litter size was 1.21 respectively.

For the fine mapping and identification of polymorphism in the BMP15 gene, 29 pairs of primers were used for amplification to cover the whole region of the gene. Amplified product was sequenced and aligned to identify the polymorphism. Sequence of sheep breed was aligned with accession # AF236078 for exon-1 and accession # AF236079 for exon-2. Nucleotide sequence of BMP15 gene (Genebank accession # AF236078 for exon-1 and accession # AF236079 for exon-2) was reported for *Ovis aries* at NCBI so the different accession number for sheep sequence alignment was applied. Investigated polymorphism, deletion and insertion at the region of BMP15 gene in Balochi are presented in Table II. Insertion of three nucleotide (CTT) at the position of 171 nucleotide was identified. Deletion of three nucleotides at the position of 3477-3479 was also observed. Insertion of the three series nucleotide (CTT) was in the exonic region while deletion of three series nucleotide was in the intronic region. These series type insertions and deletions were surprising. This novel insertions and deletions in this gene have not been reported earlier. Change in nucleotide at position 3296 from C > T was in intronic region and it was the transition while polymorphism at 4851 position (T > G) was also in intronic region but it was the transversion. Statistical analysis of identified polymorphism in BMP15 gene of the Balochi sheep revealed that SNP, BMS1 was highly significant (p= 2.40E-04). SNP, BMS3-5, also showed the significant results while SNP, BMS2 and BMS6 were found non significant. Electropherogram of the identified polymorphism in balochi sheep were also presented and change in nucleotide was marked with arrow sign. Nucleotide alignment of Balochi sheep with query was aligned and change in nucleotide was mention. Change in the amino acid affects the molecular weight of protein and ultimately may alter the expression of the gene at cellular level. Molecular weight of BMP15 protein (AF236078-9) was 44897.68 daltons while molecular weight of subject protein (balochi sheep) was 45010.83 daltons. BMP15 protein of the balochi sheep was 113.15 daltons heavier than the query protein. These results showed that this change in molecular weight may have significant effect on the expression of the gene. This appears to agree that identified SNPs in sheep shows correlation with BMP15 gene. Because of the lack of functional data and small population size used, the conclusion requires further studies on litter size in different parities. Besides the identification of SNP in conducted experiment, gene expression patterns dependent study will help to understand the molecular mechanisms of these genes to controlling the fecundity in sheep. Identified SNPs in this study can be used as genetic marker for fecundity in sheep breeds. These genetic markers can also be incorporated into genetic evaluation or artificial selection.

ACKNOWLEDGEMENTS

Special thanks are due to Directorate of Livestock Farms and Directorate of Research, Livestock and Diary Development Department, Balochistan, Superintendant Karakul Sheep Farm Maslakh at Quetta, and Superintendant Bhagnari Cattle/Balochi sheep Farm Usta Mohammad, Balochistan for helping in sample collection.

REFERENCES

ECONOMIC SURVEY OF PAKISTAN. 2012-13. Finance Division, Economic Advisor Wing, Government of Pakistan, Islamabad.

DAVIS, G.H., 2005. Major genes affecting ovulation rate in sheep. *Genet. Sel. Evol.*, **37**: 11-23.

ECKERY, D.C., WHALE, L.J., LAWRENCE, S.B., WYLDE, K.A., McNATTY, K.P. AND JUENGEL. J.L., 2002. Expression of mRNA encoding growth differentiation factor 9 and bone morphogenetic protein 15 during follicular formation and growth in a marsupial, the brushtail possum (Trichosurus vulpecula). *Mol Cell Endocrinol.*, **192:** 115-126.

GALLOWAY, S.M., McNATTY, K.P., CAMBRIDGE, L.M., LAITINEN, M.P., JENNIFER, S., JOKIRANTA, R.J., MCLAREN, K., LUIRO, K.G., DODDS, G.W.,

MONTGOMERY, J.L., BEATTIE, A.E., DAVIS, G.H. AND RITVOS, O., 2000. Mutations in an oocyte-derived growth factor gene BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.*, **25:** 279-283.

GLISTER, C., KEMP, C.F. AND KNIGHT, P.G., 2004. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differentia modulation of Smad-1 phosphorylation by follistatin. *Reproduction*, **127:** 239-254.

HALL, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.*, **41:** 95-98.

JUENGEL, J.L., HUDSON, N.L., HEATH, D.A., SMITH, P., READER, K.L., LAWRENCE, S.B., O'CONNELL, A.R., LAITINEN, M.P., CRANFIELD, M., GROOME, N.P., RITVOS, O. AND McNATTY, K.P. 2002. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biol. Reprod.*, **67:**1777-1789.

McNATTY, K.P., JUENGEL, J.L., READER, K.L., LUN, S., MYLLYMAA, S., LAWRENCE, S.B., WESTERN, A., MEERASAHIB, M.F., MOTTERSHEAD, D.G., GROOME, N.P., RITVOS, O. AND LAITINEN, M.P. 2005. Bone morphogenetic protein 15 and growth differentiation factor 9 cooperate to regulate granulosa cell function. *Reproduction*, **129:**473-480.

MORRIS, C.A., 1990. Theoretical and realized responses to selection for reproductive rate. *Proc. 4th World Congress on Genetics Applied to Livestock Production Edinburgh*, **16:** 309.

PENNETIER, S., UZBEKOVA, S., PERREAU, C., PAPILLIER, P., MERMILLOD, P. AND DALBIES-TRAN, R. 2004. Spatiotemporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol. Reprod.*, **71:**1359-1366.

RAFIQ, S. AND MUNIR, M., 1983. *Sheep and goats: practices and potentials in Balochistan. Part: IV (chaghi and Kharan District) Arid Zone Res. Inst.* Pakistan Agriculture research Council, Quetta Pakistan.

SAMBROOK, J. AND RUSSEL, D.W., 2001. *Molecular cloning: A laboratory mannual*, 3rd ed. Cold spring harbor laboratory press, New York, USA.

SOUZA, C.J., CAMPBELL, B.K., McNEILLY, A.S. AND BAIRD, D.T. 2002. Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immune histochemistry. *Reproduction*, **123:**363-369.

WILSON, T., WU, X.Y., JUENGEL, J.L., ROSS, I.K., LUMSDEN, J.M., LORD, E.A., DODDS, K.G., WALLING, G.A., McEWAN, J.C., O'CONNELL, A.R., McNATTY, K.P. AND MONTGOMERY., G.W. 2001. Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biol. Reprod.*, **64:**1225-1235.

(Received 4 July 2013, revised 21 September 2013)