

Association of a Single Codon Deletion in Bone Morphogenetic Protein 15 Gene with Prolificacy in Small Tail Han Sheep*

W. Guo^{1,2}, M. X. Chu³, X. M. Deng², J. D. Feng¹, Ning Li^{1,**} and Changxin Wu²

¹ State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100094, P. R. China

ABSTRACT : Small Tail Han Sheep has significant characteristics of high prolificacy and non-seasonal ovulatory activity and is an excellent local sheep breed in P. R. China. Recently a novel member of the transforming growth factor β (TGF β) superfamily termed bone morphogenetic protein 15 (BMP15) was shown to be specifically expressed in oocytes and to be essential for female fertility. Therefore, BMP15 is a candidate gene for reproductive performance of Small Tail Han Sheep. The whole genomic nucleotide sequence of BMP15 gene in Small Tail Han Sheep was searched for polymorphisms by PCR-SSCP and direct sequencing, and only one polymorphism was found. The polymorphism was a result of a 3 base pair deletion, which eliminated a single Leu codon (CTT). The allelic frequencies for A (without deletion) and B (with a codon deletion) are 0.73 and 0.27 respectively. The effects of BMP15 genotype on litter size were evaluated using the least squares model. This indicated that there was a significant association between litter size of Small Tail Han Sheep and a deletion in BMP15 gene ($p=0.02<0.05$). Small Tail Han Sheep ewes with AA and AB genotype produce on average 0.5 and 0.3 more lambs per litter than those ewes with BB genotype. (*Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 11 : 1491-1495*)

Key Words : Sheep, Prolificacy, Bone Morphogenetic Protein 15 Gene, PCR-SSCP

INTRODUCTION

Reproductive traits are of primary interest in farm animals because they play a major role in efficiency of livestock production. The most important reproductive trait in sheep is litter size, a typical quantitative trait that is controlled by both genetic and environmental factors. Although improving litter size is one of the main priorities of sheep industry, only limited progress has been made to date. This can be attributed to the fact that the trait is lowly heritable, expressed relatively late in life and is sex-limited. Use of polymorphic QTL or associated genetic markers in selection programs would be particularly advantageous for this trait, and some attempts have already been made by Pelletier et al. (2000) and Notter et al. (2003) to identify these.

Bone morphogenetic protein 15 (BMP15), also known as growth differentiation factor 9B (GDF9B), is a member of the transforming growth factor β (TGF β) superfamily and is specifically expressed in oocytes. This gene has been shown to be expressed during early oocyte development in

the transitory or primary follicle of human, mouse and rat (Jaatinen et al., 1999; Kang et al., 2002). Ovine BMP15 gene is similar to human, mouse and rat orthologues, with typical features of genes encoding other members of the TGF β superfamily. BMP15 gene structure was determined in mouse, human and sheep separately. In sheep, the full-length 1,179 bp sequence of 2 exons, separated by an intron of approximately 5.4 kb, encodes a pre-propeptide of 393 amino acids. A predicted signal peptide of 25 amino acids (Galloway et al., 2000) precedes a 244 amino-acid pro-region and a putative 125 amino-acid carboxy-terminal mature peptide region beyond the RRAR protease cleavage site. The ovine BMP15 coding region sequence is 82.9% homologous to that of human, 78.8% to that of mouse and 78.4% to that of rat.

Galloway et al. (2000) showed that BMP15 is essential for ewe fertility and that natural mutations in an ovary-derived factor can cause both increased ovulation rate and infertility phenotypes in a dosage-sensitive manner. Jaatinen et al. (1999) concluded that this novel TGF- β family member is likely to be a secreted growth factor that may regulate folliculogenesis at similar developmental stages as growth differentiation factor 9 (GDF-9) (Aaltonen et al., 1999; Bodensteiner et al., 1999,2000). Without BMP15, oocytes continue to grow in the absence of granulosa cell proliferation until they are unable to be supported by the residual granulosa cells, whereupon they degenerate (Braw-Tal et al., 1993; Smith et al., 1997). Although lack of BMP15 blocks follicular growth in homozygotes, inactivation of only one copy of BMP15 increases ovulation rate (Davis et al., 1991,1992). This may be because a larger proportion of antral follicles contain granulosa cells that

* Supported by National Natural Science Foundation of China (No. 30300248, No. 30140004, No. 39770541), National Major Basic Research Development Program (G20000161), China High-Tech Research Development Program (2001AA213061).

** Corresponding Author: Ning Li. Tel: +86-10-6289-3904, Fax: +86-10-6289-3904, E-mail: ninglbau@public3.bta.net.cn

² College of Animal Science and Technology, China Agricultural University, Beijing 100094, P. R. China.

³ Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100094, P. R. China.

Received December 26, 2003; Accepted July 3, 2004

Table 1. Primer pairs, expected size, location and programs used for amplification of *BMP15* gene

Size and location	Primer sequences (5'→3')	PCR programs
230 bp exon1:181-411	F1: 5'-CGTTATCCTTTGGGCTTTTATC-3' R1: 5'-CGGCTTCCTCTGCTGCTTG-3'	95°C for 5min. 95°C for 45s, 62°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.
261 bp exon1:393-654	F2: 5'-CAAGCAGCAGAGGAAGC-3' R2: 5'-GCCTGACAGAAAACACTGAC-3'	95°C for 5 min. 95°C for 45s, 54°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.
300 bp exon2:360-660	F3: 5'-GCTTTGCTCTTGTTCCC-3' R3: 5'-GCCCCTTGATTCAG-3'	95°C for 5 min. 95°C for 45s, 54°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.
309 bp exon2:641-950	F4: 5'-GCTCTGGAATCACAAGGGG-3' R4: 5'-GTGGAGGGAACACTGGTTAC-3'	95°C for 5 min. 95°C for 45s, 62°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.
277 bp exon2:785-1062	F5: 5'-GAGTGTTTCAGAAGACCAAACC-3' R5: 5'-GACCATAGTGTAGTACCCGAG-3'	95°C for 5 min. 95°C for 45s, 64°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.
254 bp exon2:1005-1259	F6: 5'-CATCTCTATACCCCAAACACTAC-3' R6: 5'-TCACCTTGCCGTCACCTG-3'	95°C for 5 min. 95°C for 45s, 60°C for 45s, 72°C for 45s with 35 cycles. 72°C for 7 min.

F stands for forward primer, R stands for reverse primer. The location numbers are based on the sequences of AF236078 and AF236079 in GenBank,

become responsive to LH (Shackell et al., 1993). There are two explanations for this phenomenon. One explanation is that 50% of normal levels of the active peptide allows more than one follicle to be selected for ovulation by reducing the number of mitotic divisions and the amount of steroid or inhibin produced by each follicle and delaying suppressive effects on plasma FSH concentrations (Henderson et al., 1988). Another possibility is that reduced levels of biologically active BMP15 affects the actions of other oocyte-derived growth factors on granulosa cell proliferation and differentiation such as GDF-9 (McGrath et al., 1995), a closely related member of the TGF- β superfamily. GDF-9 is known to influence granulosa cell function and it is essential for follicular growth (Dong et al., 1996; Hayashi et al., 1999; Elvin et al., 1999,2000). The objectives of the present study were firstly to analyze the polymorphism of BMP15 gene, and then to examine the relationship between BMP15 gene and high prolificacy in Small Tail Han Sheep.

MATERIALS AND METHODS

Genomic DNA preparation

The blood samples of 240 ewes from Jiaxiang Small Tail Han Sheep Breeding Farm (also named National Small Tail Han Sheep Breed Conservation Farm) of Shandong Province, P. R. China were collected and anticoagulated in ACD (acid citrate dextrose). Each of the ewes had records of litter size (number of lambs born per ewe lambing) covering 2 parities. There had been no selection on litter size or other fertility traits performed in the flock over previous years. The genomic DNA was prepared from whole blood by a routine protocol (Sambrook et al., 1989), and then dissolved in TE buffer and stored in -20°C.

PCR amplification of sheep genomic DNA

Primer nucleotide (nt) sequences were synthesized by referring to the GenBank nucleotide database (Galloway et

al., 2000). Genomic DNA fragments of ovine BMP-15 were amplified by polymerase chain reaction (PCR) using primers listed in Table 1. Amplifications were performed as follows: 25 μ L of each PCR mixture containing 50-100 ng of total DNA, 25 pmol of each primers, 2.5 μ L of 10 \times Taq polymerase buffer solution (2.5 mM MgCl₂) and 1 U of Taq polymerase for PCR was incubated in the thermal cycler (PE Amp2400, Peking-Elmer, USA).

PCR-SSCP analysis

The PCR products were loaded onto a polyacrylamide gel for electrophoresis in 1 \times TBE buffer at 140 volt for 14-16 hours at 4°C. The gel was stained with silver nitrate (silver staining) to identify single strand conformation polymorphisms (SSCP) or to score genotypes. Only PCR products amplified by the F1/R1 primer combination showed polymorphism.

Sequence analysis

PCR products from each (6) of the primer pair combinations were purified, ligated into plasmid pGEMT-Easy vector (Promega Corp.), and transformed into *E. coli* DH5 α competent cells. The recombinant plasmid DNA was isolated and sequenced on ABI 377 DNA sequencer. Each genotype was sequenced at least two times. At the same time, we performed protein structure prediction of BMP15.

Statistical analysis

The least squares model according to the characteristics of experimental material was constructed:

$$y_{ij} = \mu + G_i + e_{ij}$$

where y_{ij} is phenotypic value of litter size (first parity and second parity), μ is population mean, G_i is the fixed effect of the i^{th} genotype of BMP15 gene ($i=1, 2, 3$); and e_{ij} is random residual value of each observation. Calculations were achieved using Proc GLM (General Linear Model) of

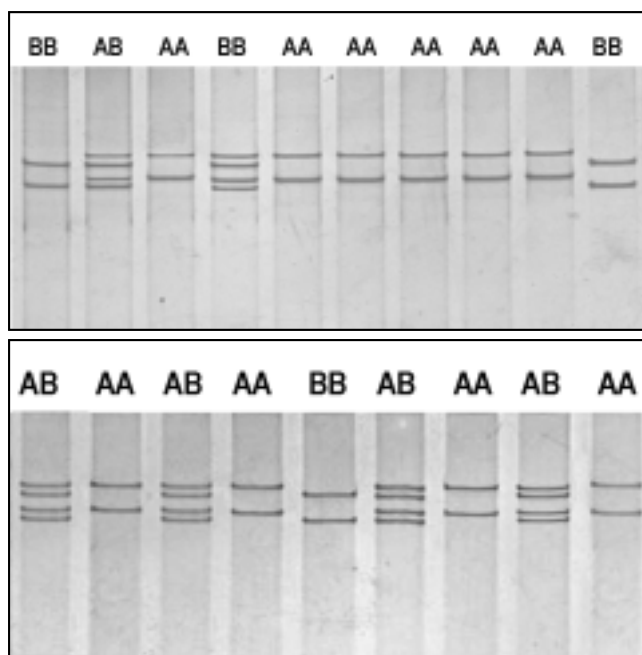


Figure 1. SSCP analysis of PCR products amplified by F1/R1 primer for different Small Tail Han ewes.

Table 2. Genotype frequency and allelic frequency of the locus revealed by F1/R1 primer in Small Tail Han Sheep

Genotype frequency			Allelic frequency	
AA	AB	BB	A	B
0.5333	0.3917	0.0750	0.7292	0.2708

SAS (6.12 version) (Biswas et al., 2003; Chu et al., 2003a,b).

RESULTS

PCR-SSCP analysis of the exon 1 and 2 of ovine *BMP15* gene

Sheep genomic DNA was amplified using six pairs of primers, that covered the entire coding sequence of BMP 15. We found that only PCR products amplified by the first primer pair produced polymorphic patterns (Figure 1). The A allele was defined as the nucleotide sequence of BMP 15 without the Leu codon deletion, and the B allele as the sequence with the single Leu codon deletion.

Sequence analysis of the polymorphism fragments and molecular modeling of BMP15

The nucleotide sequence amplified by the first primer pair (F1/R1 in Table 1) is 230 bp and covers about the first half of exon 1 of sheep BMP-15 gene. We sequenced the DNA fragments recovered from all three genotypes and identified a 3 base pair deletion of a Leu codon (CTT) in a region encoding a signal peptide (Figure 2), at position 262 to 264 of exon 1 (the mutation is deposited in GenBank with accession number: AF236079). Since we were unable

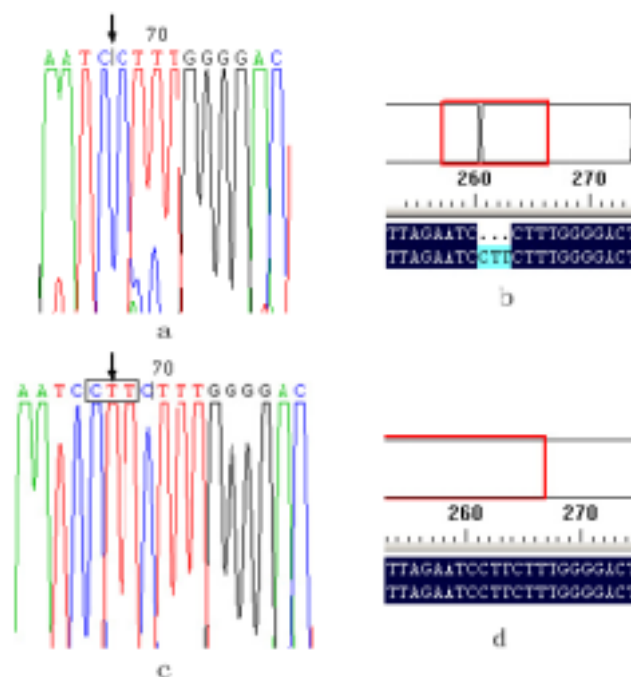


Figure 2. Nucleotide sequence comparison of the PCR products amplified by F1/R1 primer; a. nt sequence of genotype BB (arrow points to the site of CTT deletion); b. BLAST result of the nt sequence of genotype BB in this study with the previously published nt sequence in GenBank; c. nt sequence of genotype AA (arrow's position is consistent with nt sequence in GenBank); d. BLAST result of nt sequence of genotype AA in this study with nt sequence in GenBank.

to identify further polymorphisms using other primer pairs, we decided to sequence PCR products amplified by F2/R2, F3/R3, F4/R4 and F5/R5 primer pairs (Table 1) from 6 randomly selected ewes, which covered an intact exon 1 and most of exon 2, 1,282 bp in total. No further polymorphisms were identified. At the same time, we predicted molecular modeling of BMP15 using the experimentally determined tertiary structure of BMP2 (Kirsch et al., 2000) and BMP7 (Griffith et al., 1996) as a template (Kelley et al., 2000). The result indicated that this modeling would not show any changes associated with that polymorphism.

Association analysis between litter size of Small Tail Han sheep and BMP-15 gene

The gene and genotype frequencies at the BMP15 locus in Small Tail Han Sheep population studied are presented in Table 2. Table 2 indicates that the frequency difference between A and B is significant. The χ^2 value for different genotypes of BMP-15 gene in Small Tail Han Sheep is $0.0166 < \chi^2$ (df=1, 0.05)=3.84, $p > 0.05$, indicating that the genotype frequencies at this locus have already achieved Hardy-Weinberg equilibrium.

Analysis of variance showed that the BMP15 genotypes have significant effect on litter size of the second parity

Table 3. Least squares mean (LSM) and standard error (SE) of litter size for each genotype in Small Tail Han Sheep

Genotype	Sample size	First parity		Second parity	
		LSM	SE	LSM	SE
AA	128	2.10	0.06	2.60 ^a	0.08
AB	94	2.08	0.07	2.40 ^{ab}	0.09
BB	18	1.80	0.17	2.10 ^b	0.21

Means with the different superscripts within the same column differ significantly ($p < 0.05$).

($p = 0.04 < 0.05$), but no significant effect on the first parity ($p = 0.25 > 0.05$). The least square means and the standard errors for litter sizes of the different genotypes in Small Tail Han Sheep are presented in Table 3. It shows that for the first parity, the least squares mean litter size is the highest in AA genotype, followed by AB and BB genotype, but is not statistically significantly different (between AA and AB $p = 0.84 > 0.05$, between AB and BB $p = 0.13 > 0.05$, between AA and BB $p = 0.10 > 0.05$). For the second parity, the trend in the least squares mean corresponding to the different genotypes are the same as for the first parity. Differences in litter size between AA and AB genotypes, and BB and AB genotypes is not significant ($p = 0.09 > 0.05$; $p = 0.18 > 0.05$, respectively), but the difference between AA and BB genotypes is significant ($p = 0.02 < 0.05$). The average numbers of lambs for Small Tail Han ewes with AA genotype and AB genotype are 0.5 and 0.3 more than that for those with BB genotype respectively.

DISCUSSION

By performing homology-based molecular modeling of the BMP15 monomer using the experimentally determined tertiary structures of BMP2 (Kirsch et al., 2000) and BMP7 (Griffith et al., 1996) as a template, the results indicated that the overall structure of BMP15 is similar to other TGF β superfamily members, being an α -helix and two fingers, each with two anti-parallel β -strand segments. Because the Leu deletion occurs in the signal peptide of the N-terminus region (Galloway et al., 2000), we conjectured that the Leu deletion might have an effect on BMP15 secretion.

Galloway et al. (2000) reported that a single C-T transition at nucleotide position 67 of the mature peptide-coding region of FecX^H carriers introduces a premature stop codon in the place of glutamic acid (Q) at amino-acid residue 23 (residue 291 of the unprocessed protein). Such premature truncation probably results in complete loss of BMP-15 function. A different polymorphism, a single T-A transition occurs in FecX^L carriers at nucleotide position 92 of the mature peptide. The mutation substitutes a valine (V) with an aspartic acid (D) residue at position 31 (residue 299 of unprocessed protein). The FecX^L mutation may produce the observed phenotype by impairing the ability of BMP15 to form dimers. Both Inverdale and Hanna sheep often give birth to twins and triplets but Small Tail Han Sheep usually give birth to twins and quadruplets. Nevertheless, we only

detected a single leu codon (CTT) deletion at L10 or L11 in the signal sequence and didn't identify any other similar transitions in Small Tail Han Sheep as observed in Inverdale sheep, and with the evidence collected to date we cannot elucidate the molecular mechanism of how BMP 15 influences litter size. However, the results of this study properly indicate that we did identify a genetic marker for evaluation of litter size in Small Tail Han Sheep, and a codon deletion has been discovered in the coding region for the signal peptide of BMP 15. Further investigation is required to determine if this deletion has a determinate effect on protein function.

IMPLICATIONS

The polymorphism we have identified in the coding region of signal peptide of BMP 15 could be a potential genetic marker for litter size in Small Tail Han Sheep. More tests using larger ewe samples are needed in Small Tail Han Sheep or even in other sheep breeds to verify the association effect identified in this study. This will allow us to further understand that a functional BMP15 gene has the true magnitude and significance of the positive effect on female reproductive performance in sheep.

REFERENCES

- Aaltonen, J., M. P. Laitinen, K. Vuojolainen, R. Jaatinen, N. Horelli-Kuitunen, L. Seppa, H. Louhio, T. Tuuri, J. Sjoberg, R. Butzow, O. Hovata, L. Dale and O. Ritvos. 1999. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *J. Clin. Endocrinol. Metab.* 84(8):2744-2750.
- Biswas, T. K., T. K. Bhatta Charya, A. D. Narayan, S. Badola, Pushpendra Kumar and Arjara Sharma. 2003. Growth Hormone Gene polymorphism and its effect on birth weight in cattle and buffalo. *Asian-Aust. J. Anim. Sci.* 16(4):494-497.
- Bodensteiner, K. J., C. M. Clay, C. L. Moeller and H. R. Sawyer. 1999. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/ differentiation factor-9 in ovine and bovine ovaries. *Mol. Endocrinol.* 60:381-386.
- Bodensteiner, K. J., K. P. McNatty, C. M. Clay, C. L. Moeller and H. R. Sawyer. 2000. Expression of growth and differentiation factor-9 in the ovaries of fetal sheep homozygous or heterozygous for the Inverdale prolificacy gene (FecXI)1. *Biol. Reprod.* 62:1479-1485.
- Braw-Tal, R., K. P. McNatty, P. Smith, D. A. Heath, N. L. Hudson, D. J. Phillips, B. J. McLeod and G. H. Davis. 1993. Ovaries of

- ewes homozygous for the X-linked Inverdale gene (FecXI) are devoid of secondary and tertiary follicles but contain many abnormal structures. *Biol. Reprod.* 49(5):895-907.
- Chu, M. X., J. Z. Wang, A. G. Wang, N. Li and J. L. Fu. 2003. Association analysis between five microsatellite loci and litter size in Small Tail Han sheep. *Asian-Aust. J. Anim. Sci.* 16(11):1555-1559.
- Chu, M. X., C. L. Ji and G. H. Chen. 2003b. Association between PCR-RFLP of melatonin receptor 1a gene and high prolificacy in Small Tail Han sheep. *Asian-Aust. J. Anim. Sci.* 16(12):1701-1704.
- Davis, G. H., J. C. McEwan, P. F. Fennessy, K. G. Dodds and P. A. Farquhar. 1991. Evidence for the presence of a major gene influencing ovulation rate on the X chromosome of sheep. *Biol. Reprod.* 44:620-624.
- Davis, G. H., J. C. McEwan, P. F. Fennessy, K. G. Dodds, K. P. McNatty and W. S. O. 1992. Infertility due to bilateral ovarian hypoplasia in sheep homozygous (FecXI FecXI) for the Inverdale prolificacy gene located on the X chromosome. *Biol. Reprod.* 46(4):636-640.
- Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu and M. M. Matzuk. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, 383(6600):531-535.
- Elvin, J. A., A. T. Clark, P. Wang, N. M. Wolfman and M. M. Matzuk. 1999. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol. Endocrinol.* 13:1035-1048.
- Elvin, J. A., C. N. Yan and M. M. Matzuk. 2000. oocyte-expressed TGF- β -superfamily members in female fertility. *Mol. Cell. Endocrinol.* 159:1-5.
- Galloway, S. M., K. P. McNatty, L. M. Cambridge, M. P. Laitinen, J. L. Juengel, T. S. Jokiranta, R. J. McLaren, K. Luiro, K. G. Dodds, G. W. Montgomery, A. E. Beattie, G. H. Davis and O. Ritvos. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.* 25(3):279-283.
- Griffith, D. L., P. C. Keck, T. K. Sampath, D. C. Rueger and W. D. Arlson. 1996. Three-dimensional structure of recombinant human osteogenic protein 1: Structural paradigm for the transforming growth factor b superfamily. *Proc. Natl. Acad. Sci. USA.* 93:878-883.
- Hayashi, M., E. A. McGee, G. Min, C. Klein, U. M. Rose, M. van Duin and A. J. Hsueh. 1999. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* 140(3):1236-1244.
- Henderson, K. M., L. C. Savage, R. L. Ellen, K. Ball and K. P. McNatty. 1988. Consequences of increasing or decreasing plasma FSH concentrations during the preovulatory period in Romney ewes. *J. Reprod. Fertil.* 84:187-196.
- Jaatinen, R., M. P. Laitinen, K. Vuojolainen, J. Aaltonen, H. Louhio, K. Heikinheimo, E. Lehtonen and O. Ritvos. 1999. Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B. *Mol. Cell. Endocrinol.* 156(1-2):189-193.
- Kang, W. J., D. S. Seo and Y. Ko. 2002. Ovarian TGF- β 1 regulates yolk formation which involve in egg weight of Korean Native Ogol Chicken. *Asian-Aust. J. Anim. Sci.* 15(11):1546-1552.
- Kelley, L. A., R. M. MacCallum and M. J. Sternberg. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* 299:499-520.
- Kirsch, T., W. Sebald and M. K. Dreyer. 2000. Crystal structure of the BMP-2-BRIA ectodomain complex. *Nat. Struct. Biol.* 7:492-496.
- McGrath, S. A., A. F. Esquela and S. J. Lee. 1995. Oocyte-specific expression of growth/differentiation factor-9. *Mol. Endocrinol.* 9:131-136.
- Notter, D. R., N. E. Cockett and P. Chemineau. 2003. Evaluation of melatonin receptor 1a as a candidate gene influencing reproduction in an autumn-lambing sheep flock. *J. Anim. Sci.* 81:912-917.
- Pelletier, J., L. Bodin, E. Hanocq, B. Malpoux, J. Teyssier, J. Thimonier and P. Chemineau. 2000. Association between expression of reproductive seasonality and alleles of the gene for Mel1a receptor in the ewe. *Biol. Reprod.* 62:1096-1101.
- Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, USA.
- Shackell, G. H., N. L. Hudson, D. A. Heath, S. Lun, L. Shaw, L. Condell, L. R. Blay and K. P. McNatty. 1993. Plasma gonadotropin concentrations and ovarian characteristics in Inverdale ewes that are heterozygous for a major gene (FecXI) on the X chromosome that influences ovulation rate. *Biol. Reprod.* 48(5):1150-1156.
- Smith, P., W. S. O., K. A. Corrigan, T. Smith, T. Lundy, G. H. Davis and K. P. McNatty. 1997. Ovarian morphology and endocrine characteristics of female sheep fetuses that are heterozygous or homozygous for the Inverdale prolificacy gene (FecXI). *Biol. Reprod.* 57(5):1183-1192.