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Polymorphism and structure of exon 2 of caprine melatonin receptor 1b gene and its relations to fertility and seasonal estrus*

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The first description is presented of nucleotide sequence of exon 2 of caprine melatonin receptor 1b (MT2). No polymorphisms of *MT2* gene were detected between high fertility and year-round estrous goat breeds and low fertility and seasonal estrous goat breeds. It is likely that exon 2 of *MT2* gene is not associated with fertility or reproductive seasonality in goat breeds. The nucleotide sequence of exon 2 of *MT2* gene of Jining Grey goats shows much closer phylogenetic relation to the *MT2* of sheep (97%) and cattle (94%) than to that of pig (84%), human (80%) and mouse (74%). A rather high nucleotide identity (62-64%) with the melatonin receptor 1a (MT1) of goat, sheep, human and mouse was also found. The caprine *MT2* contains the same NAXXY motif in transmembrane 7 as the other melatonin receptors. Both DRY and CYVCR motifs were detected just downstream from its third transmembrane domain (the same as in sheep and cattle) rather than NRY and CYICH found in other melatonin receptor groups.

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Melatonin is a hormone produced rhythmically by the pineal gland [Tosini and Menaker 1998, von Gall *et al*. 2002, Pevet 2003]. It has two major biological effects on animals [Malpaux *et al* 2001, Poirel *et al* 2002]. First is regulation of circadian rhythms and reproduction changes in seasonally reproducing (monoestric) mammals [Reppert *et al*. 1994, Weaver and Reppert 1996, Barrett *et al*. 1997]. The circadian effects of melatonin appear to be mediated by melatonin receptors of the hypothalamic suprachiasmatic nucleus (SCN), the site of a circadian clock [Dubocovich *et al* 1996, Weaver and Reppert 1996, McArthur *et al*. 1997, Migaud *et al*. 2005, Notter and Cockett 2005], and the reproductive effects mediated by melatonin receptors in the hypophyseal *pars tuberalis* (PT) − [Reppert *et al* [1994]. Second, melatonin administration can alter the timing of circadian rhythms in many vertebrates, including humans [Ebisawa *et al*. 1999]. The circadian effect of melatonin has led to therapeutic applications, including the use of the hormone to treat jet lag and some circadian-based sleep disorders [Ebisawa *et al*. 1999].

Melatonin exerts its effects through binding to pharmacologically specific, high-affinity, G-protein-coupled receptors [Vanecek 1988, Roca *et al*. 1996, Vanecek Pandi-Perumal *et al*. 2006]. Molecular cloning in mammals had identified two high affinity melatonin receptor subtypes − the melatonin receptor 1a (MT1) and 1b (MT2) − Reppert *et al*. [1995a]. A third high affinity melatonin receptor subtype, the melatonin receptor 1c (MT3), has been cloned from zebrafish, *Xenopus*, and chickens, but not from mammals [Reppert *et al*. 1995b, Ebisawa *et al*. 1994].

The MT1 and MT2 have similar binding properties and pharmacological characteristics, and both are G-protein coupled receptors, having seven predicted transmembrane α-helical domains. Unique features of the known melatonin receptor groups include a NRY motif downstream from the third transmembrane domain (TM), followed by sequence CXXCH, and a NAXXY sequence in transmembrane 7 [Reppert *et al*. 1994, 1995a, Barrett *et al*. 2003].

The *MT2* gene encodes a nonfunctional receptor and *MT1* gene encodes a functional receptor in Siberian hamster [Weaver *et al*. 1996]. The *MT2* gene encodes a functional receptor in both rats and mice [Weaver *et al*. 1996]. The *MT1* mediates the reproductive and circadian responses to melatonin in humans [Reppert *et al*. 1994] and in Siberian hamsters [Weaver *et al*. 1996]. The MT2 is not indispensable for reproductive and circadian responses to melatonin in Siberian hamsters [Weaver *et al*. 1996].

Whether a link exists between fertility or reproductive seasonality and the structure of the *MT2* gene in goat breeds is worth analysing. For this purpose, six goat breeds were used, including two highly fertile polyestrous (year-round estrous) breeds (Jining Grey and Boer) – Tu [1989], Malan [2000] and four low fertility monoestrous (seasonal estrous) breeds (Wendeng dairy, Inner Mongolia Cashmere, Liaoning Cashmere and Angora goats) – Roberts and Reeves 1988, Tu 1989].

Material and methods

Animals and genomic DNA preparation

All procedures were performed according to authorization granted by the Chinese Ministry of Agriculture. Those involving animals were approved by the Animal Care and Use Committee at the respective institution where the investigation was conducted. All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture.

Venous jugular blood samples (10 mL per animal) were collected from 120 Jining Grey goats (Jining Grey goats conservation base, Jiaxiang County, Shandong Province, China), 46 Boer and 42 Angora goats (Qinshui Demonstration Farm, Zhengzhuang Town, Qinshui County, Shanxi Province, China), 27 Liaoning Cashmere goats (Qingshui Town, Mentougou District, Beijing, China), 40 Inner Mongolia Cashmere goats (Inner Mongolia White Cashmere Goat Breeding Farm, Etuokeqi, Ordos City, the Inner Mongolia Autonomic Region, China), and 48 Wendeng dairy goats (Wendeng City, Shandong Province, China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by the phenol-chloroform method, and then dissolved in TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0), and kept at -20°C.

Primers and PCR amplification

Five pairs of primers were designed according to exon 2 of *Homo sapiens* gene for the melatonin 1b receptor (GenBank accession number AB033598) and predicted *Bos taurus* melatonin 1b receptor mRNA (GenBank accession number XM_607095). Amplified would be 884 bp, covering from the second amino acid residue before the second TM to the carboxyl terminal in the intracellular side of human MT2 protein (*i.e*., from the 75-th amino acid to the end of carboxyl terminal). The primers were synthesized by SHANGHAI INVITROGEN BIOTECHNOLOGY Ltd. Co. (Shanghai, China).

The following primer sequences were used:

Primer 1: 182 bp, F: 5'-GGTAACCTGTTCTTGGTGAGTCT-3',
R: 5'-GCGATGGCGGTGATGTTGA-3';
Primer 2: 174 bp, F: 5'-GTGGTCGGCTCCGTCTTCAA-3',
R: 5'-CAGGGACCCCACGAAGAAGTT-3';
Primer 3: 273 bp, F: 5'-CTGGTCCTTCTGCCCAACTT-3',
R: 5'-GAAGATCACAAAGACCACAAACA- 3' ;
Primer 4: 224 bp, F: 5'-CTGAGCATGTTTGTGGTCTT-3',
R: 5'-GCAGAGATGATCCTCTTGTATT-3';
Primer 5: 182 bp, F: 5'-GGGCTCCTGAACCAGAACTT-3',
R: 5'-TGGGCATCAGGTGGGAGATT-3'.

Polymerase chain reaction (PCR) was carried out in 25 μL volume containing approximately 2.5 μL of 10×PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl [pH

8.0], 0.1% Triton X-100), 1.3-1.8 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2 mmol/L each dNTP, 2.0 μL of 10 pmol/L each primer, 3.0 μL of 50 ng/μL caprine genomic DNA, 1.0 μL of *Taq* DNA polymerase (PROMEGA, Madison, WI, USA), and the rest was ddH_2O. PCR conditions were as follows: initial denaturation at 94°C for 8 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 56-60°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 10 min on Mastercycler® 5333 (EPPENDORF AG, Hamburg, Germany).

SSCP analysis, cloning and sequencing

The steps of SSCP analysis given below followed the method of Chu *et al.* [2009]. A volume of 2 μL PCR product was transferred into an Eppendorf tube, mixed with 5 μL gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0) and 10% glycerol. The mixture was centrifuged (G=2000) and denatured at 98°C for 10 min, then chilled on ice for 5 min and loaded on 15% neutral polyacrylamide gels – (acrylamide:bisacrylamide=37.5:1). Electrophoresis was performed in 1×Tris borate (pH 8.3) EDTA buffer at 9-15 V/cm at 4°C overnight. After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed and analysed by an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (ALPHA INNOTECH Corporation, San Leandro, CA, USA).

Primer pair for exon 2 of MT2 gene in Jining Grey goat sequencing was as follows: F: 5'-GGTAACCTGTTCTTGGTGAGTCT-3' (from primer 1), R: 5'-TGGGCATCAGGTGGGAGATT-3' (from primer 5). PCR system and conditions were the same as above except for the extension at 72°C for 1 min. PCR product of the primers was separated on 1% agarose gels and recovered using Geneclean II kit (PROMEGA, Madison, WI, USA). The ligation reaction was conducted according to manufacturer instructions (PROMEGA). Each DNA fragment was then transformed into *Escherichia coli* DH5a competence cell. Positive clones of transformed cells were identified by restriction enzyme digestion. Two clones of each genotype were selected and sequenced. Each clone was sequenced twice. The target DNA fragments in recombinant plasmids were sequenced from both directions using an ABI3730 automatic sequencer (PERKIN ELMER APPLIED BIOSYSTEMS, Foster City, CA, USA) by Shanghai INVITROGEN BIOTECHNOLOGY Co. Ltd., Shanghai, China.

In order to analyse the evolutionary relationship of *MT2* with the other melatonin receptors, we aligned it with nucleotide sequences of *MT2* including sheep (DQ789246), cattle (XM_607095), pig (AJ276454), human (U25341) and mouse (AY145850) and *MT1* including goat (AF419334), sheep (AF045219), human (BC074947) and mouse (NM_008639). A phylogenetic tree was constructed using MEGA version 2.1 [Kumar *et al*. 2001], with the neighbour-joining (NJ) procedure, and support for internodes was assessed after 1,000 bootstrap (BP) resampling steps. The sequence of rat opioid receptor gene (GenBank accession number NM_001038600) was used as outgroup.

We also compared the deduced amino acid sequence of exon 2 of Jining Grey goat's *MT2* gene with the sequence of *MT2* of sheep (DQ789246), cattle (XM_607095), pig (AJ276454), human (U25341), mouse (AY145850) and *MT1* of goat (AF419334), sheep (AF045219), human (BC074947), and mouse (NM_008639).

Results and discussion

Polymorphism of *MT2* gene and its relationship with fertility or reproductive seasonality

Genomic DNA of six goat breeds was successfully amplified using five pairs of primers for exon 2 of the *MT2* gene (Fig. 1). SSCP showed no polymorphisms with five primers in six goat breeds (Fig. 2). Because neither caprine *MT2* gene nor MT2 protein is available, we numbered the sequence site in the present study according to the nucleotide sequence (GenBank accession number AB033598) and the protein sequence (GenBank accession number NP_005950) of the human MT2 (hMT2).

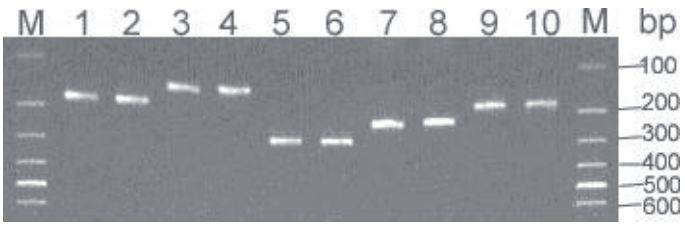

Fig.1. PCR products of five pairs of primers. M – SD002 DNA marker; lanes 1, 2 – primer 1; lanes 3, 4 – primer 2; lanes 5, 6 – primer 3; lanes 7, 8 – primer 4; lanes 9, 10 primer 5.

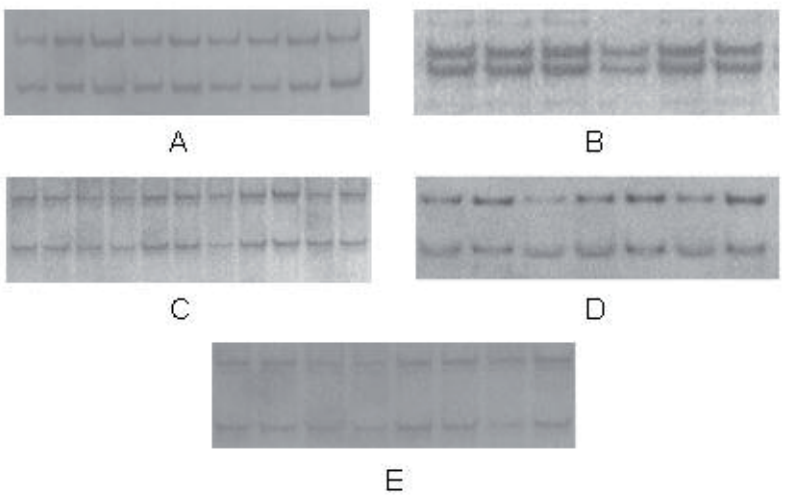

Fig. 2. SSCP analysis of PCR amplification using primers 1-5 (A-E)

Based on the sequencing results of six goat breeds including two high fecundity and polyestrous breeds (Jining Grey and Boer goats) and four low fecundity and monoestrous breeds (Wendeng dairy, Inner Mongolia Cashmere, Liaoning Cashmere and Angora), exon 2 of *MT2* gene was not found associated with fertility or reproductive seasonality.

To date, many mutations in *MT2* gene had been detected. Two missense mutations, G24E and L66F, were identified in the human *MT2* gene [Ebisawa *et al*. 2000]. The polymorphism of *MT2* gene was detected by PCR-RFLP using *Nla*III restriction enzyme in Korean rheumatoid arthritis patients and normal controls [Ha *et al*. 2005]. Qiu *et al*. [2006] identified different alleles of *MT2* gene between the adolescent idiopathic scoliosis patients and normal controls. Xiao *et al*. [2007] found 33 mutations in the *MT2* gene between high fecundity and polyestrous breeds (Small Tail Han and Hu sheep) and low fecundity and moestrous breeds (Dorset, Texel and Corriedale sheep).

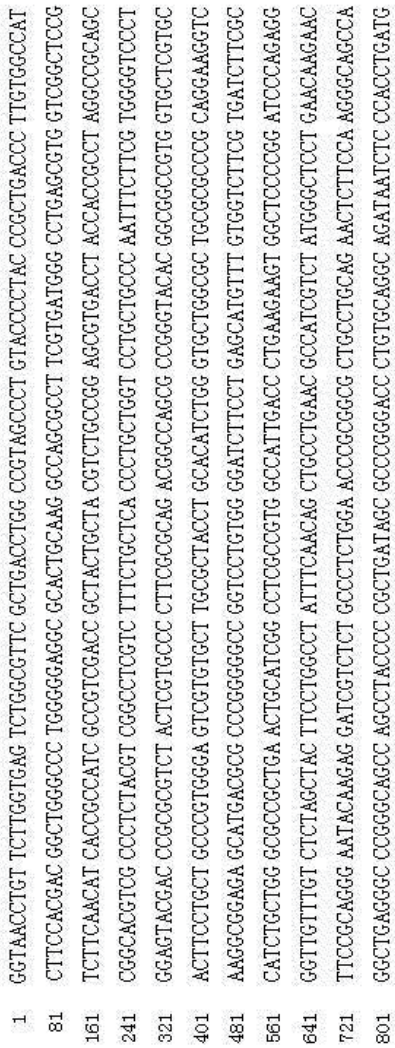

Fig. 3. Nucleotide sequence of exon 2 of *MT2* gene in Jining Grey goat.

Phylogenetic relationship of *MT2* gene among species

The nucleotide sequence of exon 2 in *MT2* gene of Jining Grey goat obtained by cloning and sequencing is shown in Fig. 3 and the caprine *MT2* gene (GenBank No. JF266705) was reported for the first time. The NJ tree (Fig. 4) shows that the caprine *MT2* is closely related to *MT2* of sheep, cattle, pig, human, mouse and diverges strongly from *MT1* of goat, sheep, human and mouse (BP = 100%). The deduced amino acid sequence (Fig. 5) of caprine MT2 is 95%, 92%, 75%, 74% and 69%, identical to sheep, cattle, pig, human and mouse MT2, respectively. On the other hand, compared with MT1, the caprine MT2 shows slightly higher identity with human (59%), goat (58%) and mouse (58%) than with sheep (57%).

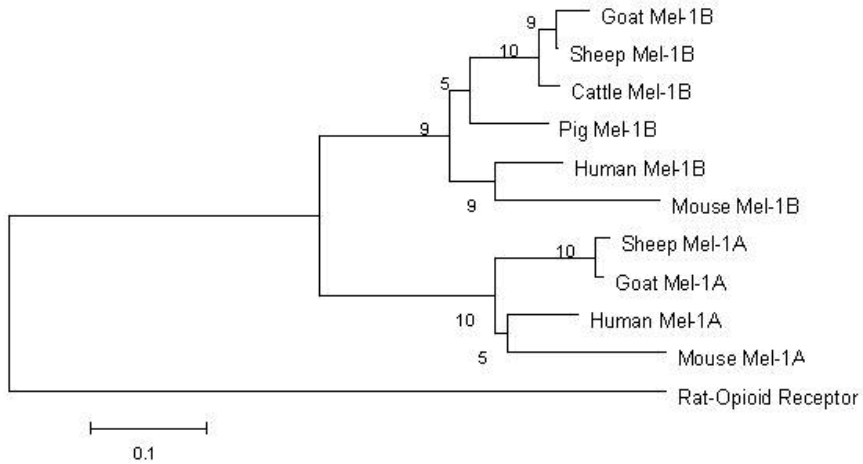

Fig. 4. Phylogenetic tree of the melatonin receptors including nucleotide sequences of the known Mel_{1a} receptors and Mel_{1b} receptors, and exon 2 of Mel_{1b} receptor of goats constructed by neighbour-joining method.

Structure of *MT2* Gene

For the caprine MT2, 43 unique amino acids were different from other known MT2. Of them, 15 amino acids were found located in TMs (2 amino acids in TM II, 1 in TM III, 4 in TM IV, 7 in TM V, 1 in TM VII). It was the same as sheep and cattle that a DRY and CYVCR sequences were immediately downstream from the third TM. It is worthwhile to note that a NAXXY sequence was in transmembrane 7, which was rather different from other melatonin receptors (Fig. 5).

Three-dimensional modeling and subsequent site-directed mutagenesis studies on G protein-coupled receptor members have identified amino acid residues in TMs 3, 5, 6 and 7 as important to receptor structure-function relationship [Barrett *et al*. 2003]. The presumed amino acid sequence of exon 2 of Jining Grey goat's *MT2* obtained in the present study extends from the second amino acid residue before the TM II to the end of inside carboxyl terminal (according to human MT2), including all the important domains mentioned above.

Any alteration of an amino acid in a protein may affect the interaction between the receptor and its ligand. In the exon 2 of ovine *MT2* gene, four in the 14 amino acid mutations were detected in the TMs and the polarity of them is congruous with that in the wild types*, i.e*. they are all hydrophobic nonpolar amino acids except that A2:T97S are hydrophilic polar amino acids, indicating the amino acids in TMs are relatively conserved. In the non-TMs, two in 10 mutations lead to polarity change (C9 and F5), that is, the nonpolar Ala to polar Ser in C9, and polar Asp to nonpolar Ala in F5, any alteration of an amino acid in a protein may affect the interaction between the receptor and its ligand [Xiao *et al*. 2007]. In the MT2, the cys113 and cys190 in the second and

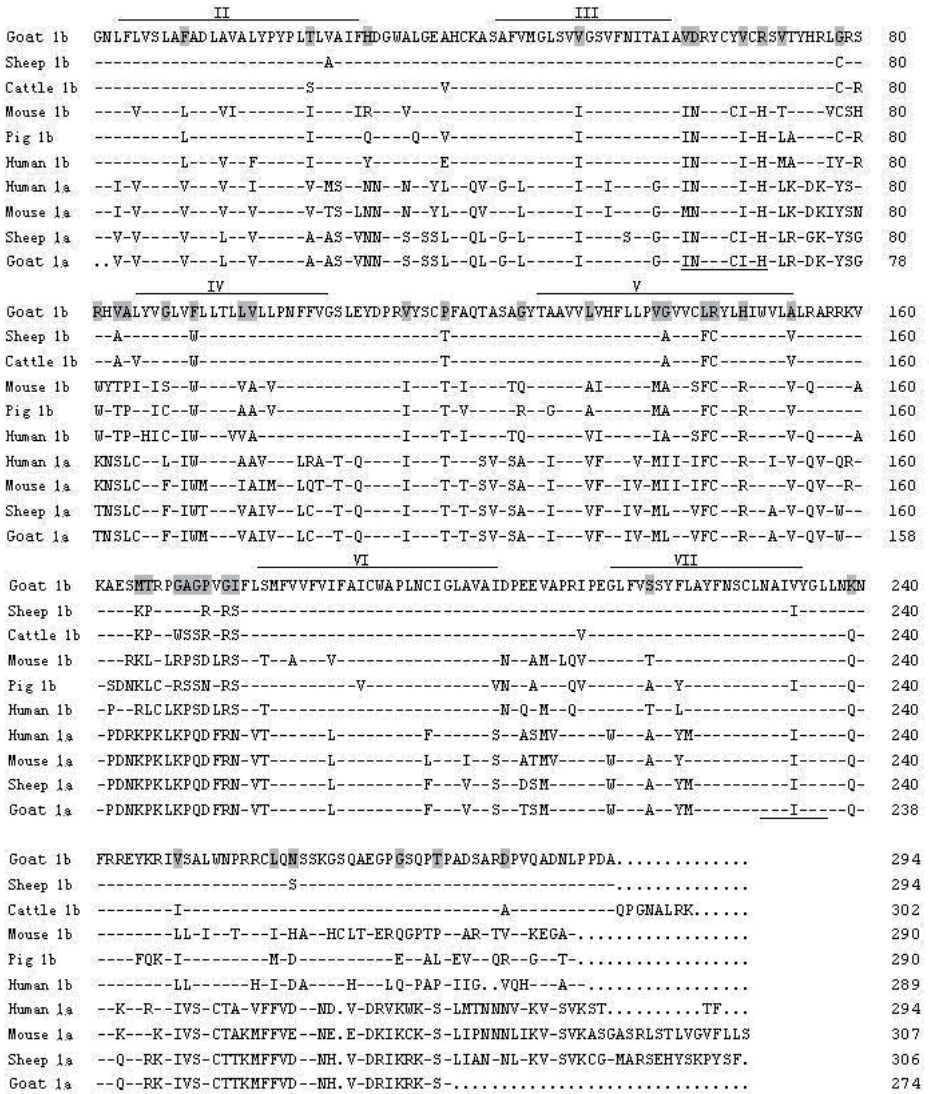

Fig. 5. Comparison of the deduced amino acid sequence of exon 2 of Jining Grey goat Mel_{1b} receptor with the known Mel_{1b} receptors and Mel_{1a} receptors.

third extracellular loop are important for the overall structure by providing a disulfide linkage between these two residues; Ser123Ala and Trp264Ala significantly reduced hMT2 expression at the cell membrane; His208Ala was found to decrease affinity for ^{125}I-Mel and melatonin and Trp264Ala increased affinity for ^{125}I-Mel; Asn175 may be

involved in the interaction with the methoxy group of melatonin [Barrett *et al*. 2003]. The Val204Ala in the fifth transmembrane resulted in total loss of binding; in the sixth transmembrane, Gly271Thr caused a substantial decrease in 2-iodomelatonin binding to hMT2, while Leu272Ala together with mutation Tyr298Ala in the seventh transmembrane completely abolished ligand binding to the receptor [Mazna *et al*. 2004]. The Asn268 and Ala275 in the sixth transmembrane as well as Val291 and Leu295 in the seventh transmembrane are also essential for 2-iodomelatonin binding to the hMT2 [Mazna *et al*. 2005]. However, no amino acid changes were found among the caprine MT2 sequence in the present study, and what affected the function of caprine MT2 is still unknown.

The Siberian hamster *MT2* gene cannot encode for a functional receptor protein, due to the presence of two nonsense mutations in the coding region of *MT2* gene [Weaver *et al*. 1996]. The *MT2* is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters [Weaver *et al*. 1996]. The MT1 gene is associated with high fertility [Chu *et al*. 2003, Notter *et al*. 2003] and reproductive seasonality [Pelletier *et al*. 2000, Notter *et al*. 2003, Chu *et al*. 2003, 2006] in sheep breeds tested. The structure of exon 2 of MT1 gene was associated with reproductive seasonality in goat [Chu *et al*. 2007]. However, the *MT2* gene was not likely to be associated with fertility or reproductive seasonality in sheep breeds tested [Xiao *et al*. 2007]. And no expression of *MT2* gene was detected in sheep by many studies [Barrett *et al*. 1997, Drew *et al*. 1998, Migaud *et al*. 2002, Xiao *et al*. 2007]. These informations implied it was likely that the exon 2 of *MT2* gene was not associated with fertility or reproductive seasonality in goats.

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