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Full Length Research Paper

Molecular cloning and characterization of a novel expressed sequence tag (EST) associated with fecundity in goats

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To screen the genes controlling the fecundity traits in goats, a DDRT-PCR technique was applied. We found a new EST which highly expressed in Chinese native prolific goat breed, Haimen goats. There exists a difference of EST expression level between the prolific and non-prolific goat breed, indicating EST might associate to fecundity in goats. A full-length cDNA with 2253 base pairs was obtained by the 3'- and 5'-RACE method based on the EST sequence encoding a protein segment of 201 amino acid residues. Tissue specific distribution and sequence analysis implicated the likely involvement of EST in the regulation of the hormones related to fecundity.

Key words: Goat, expressed sequence tag, reproduction.

INTRODUCTION

The events of the goat estrous cycle, just like other mammalians, are largely controlled by the relationship between the hypothalamic releasing hormones, gonadotropins and ovarian hormones, so-called hypothalamus-hypophyseal axis modulate. However, the seasonality and litter size of the reproductive traits are often controlled by other hormones. It is known that the FecX mutation of *GDF9* gene and FecB mutation of *BMPR1B* gene have significant effects on the litter size while *MTNR1A* gene influences out-of-season reproduction in sheep (Davis et al., 2002; Migaud et al., 2005; Davis et al., 2006; Chu et al., 2007c; Mateescu et al., 2009a). But little is known in goats. In recent years, identification of major genes for prolificacy in goats becomes more and more important to goat industry. Several genes associated with prolific traits have been identified in sheep, such as *BMP15*, *MTNR1A*, *inhibinα*, *leptin* and some microsatllites (Chu et al., 2007a; Chu et al., 2007b; Hua et al., 2007; Xu et al., 2007; Carcangiu et al., 2009b; Singh et al., 2009). Recent discoveries show that several mutations associated with the sheep prolificacy, including FecX and FecB mutations, have no relation to goat prolificacy (Hua et al., 2008).

An expressed sequence tag (EST) is a short subsequence of a transcribed cDNA. An EST sequence can be straightly related to a functional gene, which offer a rapid method to study gene sequence and gene function. At present, little EST information on reproduction trait can be gained for research in goats. In this study, we applied DDRT-PCR method to screen the genes related to goat prolificacy. An interest EST was identified in high fecundity goat breed. Full-length cDNA sequence of the EST was obtained by 3'-RACE and 5'-RACE method. Further more, tissue specific distribution was used to confirm the differences of gene expression level between two goat breeds by detecting the tissue distribution of the EST. The results indicated that the EST is indeed associated with

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Abbreviations: EST, expressed sequence tag; **cDNA,** complementary DNA; **DNase,** deoxyribonuclease; **RNase,** ribonuclease; **dNTP,** deoxyribonucleoside 5'-triphosphate; **RT,** reverse transcription; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcriptase- PCR; **PAR-2,** protease-activated receptor-2; **CNS,** central nervous systems; **Mel(1a),** melatonin receptor 1a; **Mel(1b),** melatonin receptor 1b; **EDTA,** ethylene diamine tetraacetic acid; **G-protein,** guanosine nucleotide-binding protein; **DD-PCR,** differential display- PCR; **DDRT-PCR,** differential display RT-PCR; **RACE,** rapid amplification of cDNA ends.

prolificacy in Chinese goats.

MATERIALS AND METHODS

Goat tissues preparation

All samples were collected in China. Haimen goats and Anhui white goats were collected from Haimen in Nantong City of Jiangsu Province and Wuhu City in Anhui Province, respectively. These does were selected at random from 2-year old female goats. All tissues were collected within 1 h after death, frozen in liquid nitrogen and stored at -80℃ until needed.

RNA extraction

Isolation of total RNA was performed using TRIZOL Reagent (Invitro- gen) as directed by the manufacturer's protocol. Isolated RNA was treated with DNase I at 37℃ for 30 min, and then extracted with phenol-chloroform. The quality of total RNA was evaluated by electrophoresis on 1% agarose gel containing ethidium bromide and the ratio of absorbance at 260/280 nm was measured using spectral photometer (Beckman).

Amplification of cDNA

cDNA was synthesized by RT-PCR using the 3' RACE adapter supplied with the 3'-Full RACE Core Set Ver.2.0 kit (Takara). 2 µL of 3' RACE adapter primer (5 µM) was mixed with 2 µL (1 µg/ µL) of RNA samples treated with RNase-free DNase-I and 2 µL of 10 mM dNTP. The mixture was adjusted to a final volume of 13 µL by adding DEPC treated water, incubated for 5 min at 65℃ and followed by cooling on ice for 2 min. First-strand synthesis was initiated by adding a 7 µL mixture containing 0.5 µL (200 units/ µL) M-MLV reverse transcriptase, 4 µL of 5×RT buffer, 0.5 µL of RNase inhibitor (40 U/µL) and 2 µL of 0.1 M DTT. The reverse transcription reaction was carried out at 42℃ for 1 h and then terminated by heating at 70℃ in a water bath for 15 min. The reverse transcription product of cDNA was stored at -20℃.

DD-PCR

The PCR reaction was carried out by adding 1 µL of the reverse transcription product to 24 µL of PCR solution containing 20 µmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 1 U of Taq DNA polymerase (Promega), 20 µmol/L H-T_{11}N (H-T_{11}G/ H-T_{11}C/H-T_{11}A) primer and 10 µmol/L of one of eight arbitrary primers on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany). The PCR procedure was as follows: initial denaturation at 94℃ for 5 min; followed by one cycle of denaturation at 94℃ for 30 s, annealing at 40℃ for 2 min, extension at 72℃ for 2 min; followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 2 min, extension at 72℃ for 2 min; with a final extension step at 72℃ for 5 min. The amplified fragments of DDRT-PCR were separated by electrophoresis using 8% polyacrylamide gels (acrylamide:bisacrylamide = 39:1). Then, the PCR products were visualized by silver staining, photographed and analyzed using an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Reverse northern dot blot analysis

Reverse northern dot blotting was carried out according to the instruction manual for DIG-HIGH Prime DNA labeling and detection starter kit I (Roche, Penzberg, Germany). In brief, 1 µg of template DNA was denatured by heating in a boiled water bath for 10 min and quickly chilling in an ice/water bath, then DIG-HIGH Prime was mixed and incubated at 37℃ for 1 h and the reaction was stopped by adding 2 µL of 0.2mol/L EDTA (pH 8.0). Subsequently, the RT pro- ducts were fixed on the nylon membranes positively charged by UV cross-linking, and then hybridized with DIG Easy Hyb (10 ml/100 cm^2) containing DIG-labeled DNA probe at appropriate hybridization temperature for 20 h. After hybridization and stringency washes, the membranes were incubated in 100 ml blocking solution for 30 min, 20 ml antibody solution for 30 min and detected with freshly color substrate solution in the dark after two washes with 100 ml washing buffer for 15 min. The reaction was completed after 16 h and terminated by 50 ml TE buffer for 5 min.

3' RACE and 5' RACE

The 3' - and 5' -end sequences were amplified by nested PCR using the 3'-Full RACE Core Set Ver.2.0 kit and 5'-Full RACE Kit (Takara) according to the user's manual. The primers used in this study were: 3'-Full RACE Gene Specific Inner Primer (5' –GCTTTAACCCTT TGGCATCG -3'), 3'-Full RACE Gene Specific outer Primer (5'-TCCCCACAATAAAACCAGATGCT-3'), 5'-Full RACE Gene Specific Inner Primer (5'- GGATCTTTGCAAGGGGAAAACTAC-3') and 5'-Full RACE Gene Specific outer Primer (5' –CAAGGAGCATCT GGTTTTATTGTGG- 3').

Cloning and sequencing

PCR products were separated on 1.0% agarose gels and re-extracted using Gene clean II kit (Premega, Madison, WI USA). Each DNA fragment was inserted into the pGEM-T Easy vector (Premega, Madison, WI USA). The ligation reactions were carried out at 16℃ overnight in 10 µL volume containing 1 µL PCR product, 1 µL (50 ng/ µL) pGEM-T easy vector, 1 µL (3 U/ µL) T4 ligase, 5 µL 2×ligation buffer and 2 µL distilled H_2O. The resultant was then transformed into *Escherichia coli* DH5α competent cell. Positive clones were identified by restriction enzyme digestion. Each clone was sequenced at least twice in both directions using an automatic ABI 377 sequencer (Perkin Elmer Applied Biosystems, Forster City, CA, USA) by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Tissue specific distribution

To confirm the difference of expression levels of EST in ovary tissue between two goat breeds, semi-quantity PCR method was used. The ovary and uterus were selected for the detection of expression level related to reproductive organ while muscle was selected here as non-reproductive apparatus. Semi-quantity PCR was carried out in a 20 µL reaction volume containing 20 µmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 1 U of Taq DNA polymerase (Promega), 0.5 µmol/L of each primer and cDNA obtained from 10 ng of total RNA. The PCR programme consists of 2 min at 94℃ followed by 24 cycles of 94℃ for 15 s, 60℃ for 30 s and 72℃ for 15 s. The PCR reaction was carried out on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany). A β-actin was used as the housekeeping gene for inner reference. The absolute gradient values of the PCR products were scanned on 2% agrose gel and the relative gene expression levels of multiple was calculated by the software offered by AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corpo- ration, San Leandro, CA, USA).

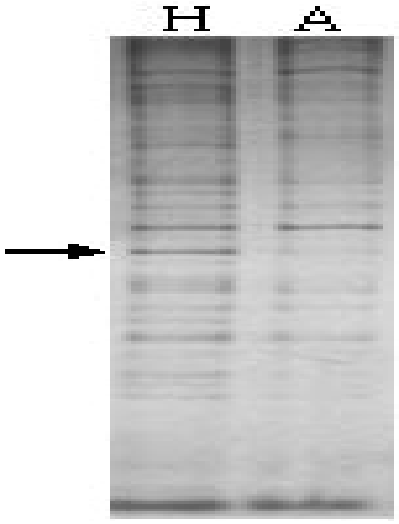

Figure 1. Partial differential display profiles from different primer combinations of goats. H represents Haimen goats for high prolificacy, A represents Auhui white goats for low prolificacy. Arrowhead point to differential expression fragments.

RESULTS

DD-PCR of Haimen goats and Anhui white goats

We displayed the different bands of two kinds of cDNA using 24 primer pairs consisting of 3 anchored primers and 8 arbitrary primers. The partial results were shown in Figure 1. The arrowhead points out the EST. At last 1195 bands in total were detected by statistics and 51 bands among them were collected and sequenced based on their differences (lack, density and size). The collected differential bands were re-amplified with the same anchor primer and arbitrary primer using recovered products as templates. However, some of the recovered bands could not be perfectly amplified because of the false positive.

Reverse northern dot blot

In order to eliminate most of the false positive bands, reverse Northern blot analysis was performed. 21 positive blots were reconfirmed from 51 differential bands of PCR amplifications by blotting amplificons onto two nylon membranes following the hybridization with cDNA probes. Partial results were shown in Figure 2. D1 is the result of the EST that we studied in this paper.

RACE and sequencing

Partial sequence, obtained by RACE method and sequenced, was compared to the NCBI's nucleotide collection (nr/nt) database using BLAST. BLAST results showed some of relatively high hits and the sequence with a highest-score was Bos taurus transmembrane emp24 domain-containing protein 2 procursor (Membrane protein P24A) (Genbank accession XM_001789741). A full-length sequence of P24A was generated by the electronic prolongation and showed in Figure 3. The computationally predicted sequence was 2253 bp in length and contained a single, continuous open reading frame (606 bp) from nucleotide 117 to 722, flanked by a 116 bp 5'-UTR, a 1531 bp 3'-UTR and a poly (A) tail. There is a GC box (nucleotide acid positions 11 - 20, boxed), two TATA boxes (nucleotide acid positions 1010 - 1016 and 2099 - 2106, bold and boxed) and four GATA boxes (nucleotide acid positions 208 - 213, 1227 - 1232, 1701 - 1795 and 2225 - 2230, shadow and boxed). A series of amino acid motifs are underlined, including three CK2 phosphorylation sites (3 - 6, 62 - 65 and 173 - 176), two MYRISTYL sites (amino acid positions 40 - 45 and 68 - 73) and four protein kinase C phosphorylation sites (amino acid positions 77 - 79, 89 - 91, 95 - 97, 102 - 104). The signal peptide (amino acid positions 18 - 22) is in shadow.

Tissue specific gene expression profile

Partial tissue-special gene expression distribution and relative expression levels in two goat breeds were shown in Figure 4. The results showed that EST was expressed in all tissues of two goat breeds included ovary, uterus and muscle. The EST expression level in ovary of Haimen goats was about 5 times higher than that in Anhui goats while there was no significant difference in uterus between two goat breeds. Interestingly, the expression level of EST in uterus of Haimen goats was a little lower than in Anhui goats, which mean that EST expression may not be synchronous in different tissues. The results showed that the EST also expressed in other tissues such as muscle besides the reproductive apparatus, indicating EST is not a special expression gene in reproductive system. It might express in all tissues.

The goal in this study is trying to find out genes affecting the fecundity in goats. DDRT-PCR method was used to screen the differential gene expression. As we all know that this technique has some defects, especially on the ratio of false band. To improve the accuracy of DDRT-PCR assay for gene detection, we somewhat improved this approach. Firstly in the screen step we made two repeats and the bands only displayed in two repeats were selected. Secondly, all detected interest bands were re- conformed by reverse Northern dot blot assay. At this step, non-specific bands were eliminated. Finally, the differen- ces of gene expression were detected again by semi- quantity method. The results of tissue-special gene expression indicated that there indeed exists a difference in ovary between two goat

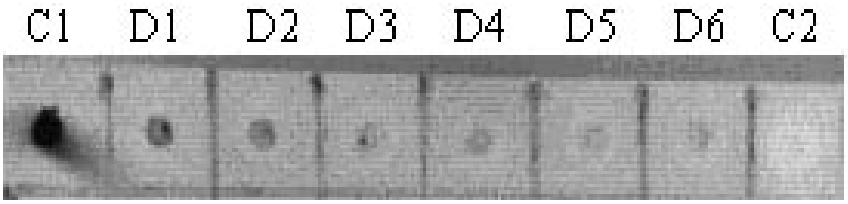

Figure 2. Partial results of reverse northern dot blot for differential display bands. C1 represents positive control. D1 is the interested band, D2 – D6 are the other bands. C2 represents negative control.

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CTCTGGAGGCCGCAGTCCCGGTCCTGGCTTCGGCCCCAGCCCCACCATGGTGACGCTTGCTGAGCTGCTG     140
                                                  M  V  T  L  A  E  L  L      8
GTGCTCCTGGCCGCCCTCCTGGCCACGGCCTCGGGCTACTTCGTCAGCATTGACGCGCATGCAGAGGAGT     210
V  L  L  A  A  L  L  A  T  A  S  G  Y  F  V  S  I  D  A  H  A  E  E  C     32
GCTTCTTCGAGCGGGTCACCTCGGGCACCAAGATGGGCCTCATCTTTGAGGTGGCCGAGGGCGGCTTCCT     280
   F  F  E  R  V  T  S  G  T  K  M  G  L  I  F  E  V  A  E  G  G  F  L     55
GGACATCGACGTGGAGATTACAGGACCTGATAATAAAGGAATTTATAAAGGAGACCGGGAGTCCAGTGGG     350
 D  I  D  V  E  I  T  G  P  D  N  K  G  I  Y  K  G  D  R  E  S  S  G      78
AAATACACATTTGCTGCTCACATGGATGGAACATATAAGTTTTGTTTTAGCAATAGGATGTCTACCATGA     420
K  Y  T  F  A  A  H  M  D  G  T  Y  K  F  C  F  S  N  R  M  S  T  M  T     102
CTCCAAAAATAGTGATGTTCACCATTGACATCGGGGAAGCCCCCAAAGGACAAGACATGGAAACAGAAGC     490
   P  K  I  V  M  F  T  I  D  I  G  E  A  P  K  G  Q  D  M  E  T  E  A     125
TCACCAGAACAAGCTAGAAGAAATGATTAATGAGCTCGCAGTGGCGATGACAGCTGTGAAGCATGAACAG     560
 H  Q  N  K  L  E  E  M  I  N  E  L  A  V  A  M  T  A  V  K  H  E  Q      148
GAATACATGGAAGTTCGAGAGAGAATACACAGAGCAATCAATGACAACACAAACAGCAGAGTGGTCCTTT     630
E  Y  M  E  V  R  E  R  I  H  R  A  I  N  D  N  T  N  S  R  V  V  L  W     172
GGTCCTTCTTTGAAGCTCTTGTTCTAGTTGCCATGACATTGGGACAGATCTACTACCTGAAGAGATTCTT     700
   S  F  F  E  A  L  V  L  V  A  M  T  L  G  Q  I  Y  Y  L  K  R  F  F     195
CGAAGTCCGGAGGGTTGTTTAAAAGCCTTTTCCCCATAATCCCAAATTCATAATTCACTGTTTACCATAA     770
 E  V  R  R  V  V  *                                                      201
TAATGTCATTAGTTTCTATGTTTTTATTTTCTGAACTATATCTTCACAGCTTGTTTCTTTGTGATTAATA     840
GATACTGGGGGAAAACACTTTTTTAGGAAAGCTATAGTGAAAATTTGACATTTGATTGGCATAAATTCAT     910
ATTTGAATTCTGCCTCCATTATACAGGCCCTCCCAAAACTCAAACACTATAAGCCGAATATAGGTGTAGA     980
GTCCTTAGTTTTTTTTCTTTTTTTTTCATTATAAAATACAGTTTGTTCAGGATAGTACTTTACTAAAATG    1050
ACTCTATTCTTGGGATCCCTTATCCTTCAGTTCAAGTCATTAGAAAGATTCATTTTGTTGAGAAACAACA    1120
ATCTGAATCTTGACATTTTCTATCCAGCTTGGTGGTAGAGCTCTATGACTAGGGTATGTGACCAGGTTGG    1190
TACTTTCAAACTTTGTCTATCTTATTTTTGCTTTAAAGATAAGACTTAAACCAACAACCTTTACCCACCC    1260
CTCCAAAGTTTCACTTTGAACCTTAGAACTCAATCACCTCCACTTAGAATTGTTGACACAAGCAGCTCAT    1330
AACCATTTTTTGGTTTCTGCCTAACCTTGTAAGAAGTCTGTTAATGCCAGTTCTCTGGGTGTTTCAGCAG    1400
ACAGTAGCTCTTTTTCTTTCTATGCTGGCGCATCCACCTTCTCTACTCTTGGCATGTAGGAGGCATGCAT    1470
TCATGTAATTGGAAAAATCTGGATTTCCGATGCCAAAGGGTTAAAGCCTCTTGGATTTCTTTGCGGTGAT    1540
TTGCAGCCACTATTTTATTTTTGATCAGTGGCATTTTGGCCACTGTTTAATTAGGGTACTGAACATCACT    1610
GTCAGTACTAGGGTTTTTGTTTTTCTTGGGTCTTTTTTGGCACATGTGAATTTTGTTTTGTATAGAACAA    1680
AACGACTTTCTTTTGGTCTCTGATAATGGGTTTAAAAATCAAGGAGCATCTGGTTTTATTGTGGGGATGA    1750
TCCGGATTATGTTGTGACTGATGTATATTGGTTACTTGTACATTTTTTTTTGGATCTTTGCAAGGGGAAA    1820
ACTACAAGTAACGAGTTTTGTATAATTAAATTTGTTACAGGTTTTCATGTTCAGGGTAAACAATTTTTCA    1890
ACCTTGGGTGAAAATACTTGCAACAGTTTAAGGTGACTGAGTTTTATCTTAGGACAACTGTTGCATGCCA    1960
ATTTGTGTGCGTGTGTGTGTGCAAACACTTCAAAATTGAGTTAAAATATGTAAATTTAAAATTGGTTGTG    2030
TATCTGATCTGCCCCAGGTTCAGTAAATAAACAGTTCTTTTTAAAACCAGTCTTGGTATTCTGTGTTTTA    2100
TATATTTATGATTAATTCACATTTGTTAAATACCAGCTTTCTAATCAAAGCTGAAATGAGACGTCATGTT    2170
TTCCCAGTAATGATGCCTATTATAAACATCTGTTACTTTTTATGTCTGTAAACTGATAATAGTAAACAC     2240
GAATCCAATTTTA                                                             2253
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Figure 3. Full-length nucleotide sequence of the EST prolongation (membrane protein P24A) with deduced amino acid sequence below. The asterisk reveals the stop codon. The signal peptide is in shadow. A series of amino acid motifs of polypeptide are underlined and nucleotide sequence motifs are boxed.

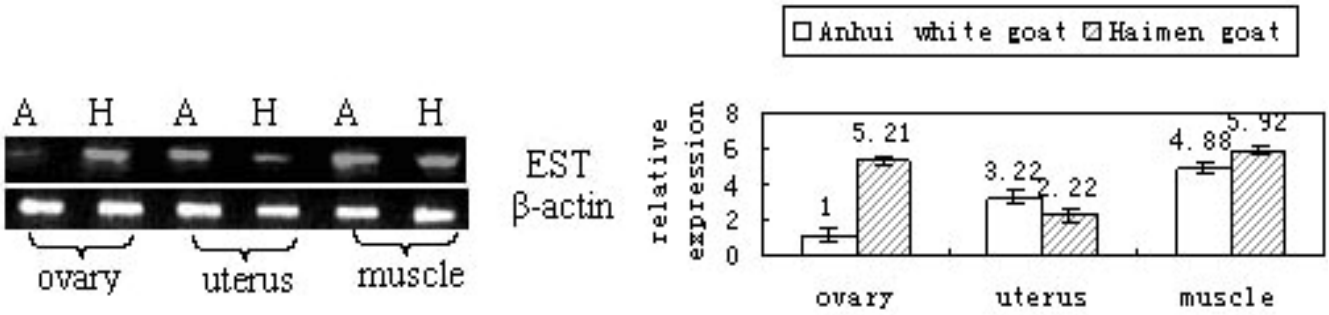

Figure 4. Tissue specific distribution and relative expression level of EST in goats. A represent Anhui white goats in China, H represents Haimen goat in China.

breeds. The EST expression level in ovary of Haimen goats is about 5 times higher than that in Anhui goats. It might be related to reproduction of goats. However, besides in reproductive aparatus the EST expression can also be detected in other tissues, for example, in muscle. It might express in all tissues.

We obtained a full-length sequence by RACE and electronic extend method. BLAST results indicated that the highest-score sequence was Bos taurus transmembrane emp24 domain-containing protein 2 procursor (membrane protein P24A) (Genbank accession XM_001 789741). As we know that the membrane protein P24A is a type I transmembrane protein, a crucial constituent of the Golgi apparatus, which is associated with protease-activated receptor-2(PAR-2) in Golgi apparatus. Also PAR-2 is a second member of the G-protein-coupled PAR family. It is irreversibly activated by trypsin or tryptase and targeted by lysosomes for degradation (Luo et al., 2007). On the other hand, two other G-protein-coupled receptors associated with fecundity have been identified in mammals. They belong to melatonin receptors, including Mel(1a) receptor and Mel(1b) receptor. Recent studies reveal that melatonin levels are higher during the night and lower during the day. Thus it can be considered as an organic informer of the annual photoperiodic trend (Lincoln et al., 2005; Carcangiu et al., 2009b). High melatonin levels, typical during autumn, have a positive influence on reproduction in small ruminants, which is called non- seasonal estrous. Melatonin functions through special melatonin receptors located in different areas of the central nervous systems (CNS), including nuclei, regulate reproduction (Sliwowska et al., 2004; Carcangiu et al., 2009b). A single base mutation of Mel(1a) receptor affected the fecundity in sheep and goats has also been identified (Hernandez et al., 2005; Chu et al., 2007a; Carcangiu et al., 2009a; Mateescu et al., 2009b). We hypothesize that the membrane protein P24A might apply the same modulation model in ovary cells with a melatonin-like function by regulating the cell function through the PAR-2, though we have no direct evidence to confirm it at this present.

Sequence analysis showed that the full sequence consisted of 116 bp 5'-UTR, 606 bp ORF from 117 to 722 bp and 1531 bp 3'-UTR. There was a GC box, two TATA boxes and four GATA boxes. There also exists a series of amino acid motifs, including three CK2 phosphorylation sites, two MYRISTYL sites, four protein kinase C phosphorylation sites and four GATA boxes. GATA regulatory motifs, originally identified a decade ago in studies of erythroid-specific gene expression, were found having an effect on animal reproduction, especially GATA-4 and GATA-6. GATA transcription factors are expressed in variety of tissues including adrenals and gonads. Steroido- genesis is regulated by GATA transcription factors. While steroidogenesis is a tightly regulated process that is dependent on the pituitary hormones. Since the regulatory regions of several steroidegenic genes contain GATA elements, it was proposed that particular GATA-4 and GATA-6 might represent novel effects of hormonal signa- ling in steroidogenic tissue (Tremblay and Viger, 2003). Transcription factor GATA also regulates inhibin in gonadal cells and the FSH cascade activates GATA-4 by TGF-β signalling pathway. Immunohistochemical studies were undertaken to determine the distribution of GATA-4 and GATA-6 in rat fetal gonad and postnatal ovary during development and pregnancy. The results showed that GATA-4 was expressed in the somatic cells of both sexes. After differentiation of the ovary and testis, GATA-4 expression continued in both ovarian and testicular somatic cells. TGF signaling cascade and GATA-4 also cooperate to regulate inhibinα expression and experiment also showed that TGF-β upregulated GATA-4 expression (Lavoie et al., 2004; Leclerc et al., 2008; McEachin et al., 2008). Therefore we propose that GATA factors can also regulate the EST expression in ovary. EST might function as a regulatory hormone, participating in whole hormone modulation in reproduction system.

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