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Molecular characterization, expression, polymorphism of NR5A2 and its relationship with litter size in Hu sheep

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ABSTRACT. *NR5A2* has been implicated in processes as diverse as steroidogenesis, cellular proliferation, ovarian follicular development, ovulation, and fertility in mammals. However, data about the relationship between *NR5A2* and prolificacy in mammals are lacking. In the present study, we identified and characterized *NR5A2* of Hu sheep, and investigated the correlation between *NR5A2* and reproductive performance. The full-length coding region was 1488 bp, and the gene was conserved in mammals. We found a positive correlation between *NR5A2* mRNA levels in the ovary and the ovulation rate and litter size of Hu sheep. We detected two single nucleotide polymorphisms (T40C and T1419C) in the coding sequence of *NR5A2*. At the third and average parity, litter size of Hu ewes with CC genotype at T40C locus was larger than those of ewes with TT

or TC genotypes; at the T1419C locus, Hu ewes with TT genotype was greater than those of ewes with CC genotype at the third parity. Our findings demonstrated that NR5A2 was associated with reproductive performance in Hu sheep, a high prolificacy breed.

Key words: Hu sheep; *NR5A2*; mRNA expression; Single nucleotide polymorphism; Reproductive performance

INTRODUCTION

NR5A2, also known as fetoprotein transcription factor (FTF) and liver receptor homolog-1 (LRH-1), is a member of the nuclear hormone receptor (NR) subfamily of transcription factors (Galarneau et al., 1996; Lu et al., 2000), and plays important roles in embryonic development, cell differentiation, bile acid metabolism and steroidogenesis (Schoonjans et al., 2002; Labelle-Dumais et al., 2007; Yazawa et al., 2010; Winkler et al., 2010). Like other NRs, *NR5A2* is comprised of four domains: DNA binding (DBD) and ligand binding (LBD) domains; a long hinger (HR), that bridges the DBD and LBD; and, a modification domain (MD) (Yumoto et al., 2012).

Human *NR5A2* was identified in 1993 (Becker-André et al., 1993), and has since been detected in rats (Boerboom et al., 2000) and bovines (Taniguchi et al., 2009) as well as other species. In adult mammals, *NR5A2* is predominantly expressed in pre-adipocyte cells and in the liver, intestine, pancreas, ovary and testis (Falender et al., 2003; Liu et al., 2003), and is particularly highly expressed in ovaries (Zhao et al., 2007; Yang et al., 2009). Further study showed that *NR5A2* is not expressed in the theca or in stromal cells, but is restricted to the granulosa and luteal cells (Fayad et al., 2004). *NR5A2* knockout mice (NR5A2-/-) died between 6.5 and 7.5 days of gestation and the fecundity of heterozygous mice (NR5A2+/-) was significantly decreased (Labelle-Dumains et al., 2007). Mice lacking *NR5A2* in granulosa cells (NR5A2 gc-/-) were sterile owning to anovulation, suggesting this gene is an essential and pleiotropic regulator of ovarian follicular development and ovulation (Duggavathi et al., 2008). Zhang et al. (2013) found mice lacking *NR5A2* exclusively in cells of the corpus luteum have ovarian dysfunction and pregnancy failure, illustrating the critical role of *NR5A2* in the uterus in mice. Kisspeptin neuron-specific *NR5A2* knockout mice (NR5A2 kiss-1-/-) have significantly reduced litter sizes (Atkin et al., 2013). In the ovary, *NR5A2* regulates genes involved in follicular development, such as *CYP11A1* (Hsieh et al., 2009); *STAR* (Taniguchi et al., 2009); *HSD3B2* (Peng et al., 2003); *INHA* (Matulis and Mayo, 2012); and *FDX1* (Imamichi et al., 2013). *NR5A2* is expressed in the anterior pituitary, and regulates key genes underlying follicular development such as those encoding GnRHR (gonadotropin-releasing hormone receptor), LHR (luteinizing hormone receptor), and FSHR (follicle stimulating hormone receptor) (Zheng et al., 2007).

To date, *NR5A2* has only been studied in a few mammals including humans (Becker-Andréet al., 1993; Bianco et al., 2014), mice (Zhang et al., 2013; Mamrosh et al., 2014), rats (Lai et al., 2014) and cattle (Taniguchi et al., 2009). Little is known about the relationship between *NR5A2* and prolificacy in mammals. In this study, we report the identification and characterization of *NR5A2* in Hu sheep, a famous breed in China characterized by high prolificacy with an average litter size of 2.29 lambs. We also determined the relationships between ovarian mRNA level, polymorphisms, and reproductive performance (ovulation rate and litter size). Here, we provide evidence for its role in mammal offspring production.

MATERIAL AND METHODS

Samples

Ten healthy Hu ewes were chosen from Hailun Sheep industry Co., Ltd., of Jiangsu province in China. Ewes were intramuscularly injected with sodium chloride forefront alcohol and the oestrus time of sheep were reorded. When the second natural estrus after 18 days, estrus was identified by the vaginal examination method, and then euthanized within 24 to 36 h after their second natural estrus. At euthanasia, the ovaries of each ewe were removed. The ovulation points (or number of corpus lutea) were determined, and the ovulation rate recorded. Heart, hypothalamus, liver, uterus, duodenum, fallopian, kidney, spleen, lung, ovary and muscle tissue samples were collected and immediately placed in liquid nitrogen, then stored at -70°C until total RNA was extracted for gene cloning and mRNA expression analysis. Ear tissue samples were obtained from 239 Hu ewes from Hailun, and transported to the laboratory on wet ice. Experiments were performed in accordance with the National Institute of Health guide for the care and use of laboratory animals (National Research Council (US) Committee for the Update of the Guide for the care and use of laboratory animals, 2011).

DNA and RNA extraction

Genomic DNA and RNA were extracted using a standard phenol-chloroform extraction protocol and a TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA), repectively. Agarose gel electrophoresis and ultraviolet spectrophotometry were used to assess RNA quality. Random primers were used for total RNA transcription, and reverse transcription (RT) product was stored at -20°C until further processing.

Cloning and sequencing

Polymerase chain reactions (PCRs) were performed in a total volume of 20 µL, containing 1 µL RT product; 2U Taq DNA polymerase (TakaRa, Shiga, Japan); 2 µL10X PCR buffer; 0.5 mM dNTP; 2.5 mM MgCl2; and 0.5 µL upstream and downstream primers (10 nM; Table 1). Amplification conditions were as follows: 94°C for 4 min; 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 35 cycles; and 72°C for 7 min. Amplification products were separated and visualized by agarose gel electrophoresis, and purified with a DNA gel extraction kit (Karroten, Nanjing, China). Recovered DNA fragments were ligated into pMD18-T vectors and transformed into *Escherichia coli* DH5α. Plasmids were extracted from positive clones with a plasmid DNA extraction kit (Karroten, Nanjing, China), and then sequenced at Shanghai Majorbio Co. (Shanghai, China).

Bioinformatics analysis

NR5A2 and deduced amino acid (aa) sequences were analyzed using DNASTAR program (DNASTAR, Inc., Madison, WI USA). Sequence alignment was performed with ClustalX 1.81 using default parameters (Thompson et al., 1997). The open reading frame (ORF) was predicted using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The online database tool ExPASy (http://us.expasy.org/cgi-bin/protparam) was used to analyze aa composition as well as the physical and chemical protein properties. Signal peptide and domain prediction were performed

using SMART (http://smart.embl-heidelberg.de). The online tool Target P1.1 server (http://www.cbs.dtu.dk/services/TargetP) was used to predict the subcellular localization of protein. The protein structure prediction server PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) was used to predict protein secondary structure. The tertiary protein structure was predicted using the online tool SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html). Phylogenetic analysis was performed using Mega 5.1 software (Tamura et al., 2011) and the neighbor-joining (NJ) method with bootstrap analysis (bootstrap test) with 1000 times repeates (bootstrap percentage, BP) (Thompson et al., 1994; Feng et al., 2015).

Table 1. Primers used in the study.

Primer	Gene	Primer sequence (5'-3')	Tm (°C)	Size (bp)	Usage
P1	NR5A2	F: TGGTTCACGGAAAGGAGGGC R: CTATGCTCGTTTTGCGTGCAG	54	443	cDNA clone
P2	NR5A2	F: AGCACGGACTTACACCTATT R: CTCCTTTCCGTGAACCACTTG	51	1019	cDNA clone
P3	NR5A2	F: ATGTCTTCTAATTCGGATACT R: GAGGCAGAGTGGATGTTCTGA	61	521	cDNA clone
P4	NR5A2	F: GCGTTGTCCTTACTGTCG R: GGCTTGGATCACCTGAGAC	60	199	Real-time PCR
P5	β-actin	F: AGCCTTCCTTCCTGGGCATGGA R: GGACAGCACCGTGTTGGCGTAGA	68	113	Real-time PCR
P6	NR5A2	F: CTCTAAACCAAAGGACTGCC R: AACACAACTGGAAAAGCAGTG	60	221	SNP genotype (PCR-RFLP)
P7	NR5A2	F1: GGCCGAGGAGTACCTGTACTAC F2: GGCCGAGGAGTACCTGTACTAT R: GTTCAATCCATGTTGCTTGG	56	213	SNP genotype (AS-PCR)

AS-PCR = allele-specific PCR; RFLP = restriction fragment length polymorphism.

Real-time PCR

The expression level of *NR5A2* in ovarian tissue of Hu sheep was assessed using a real-time PCR instrument (ABI, Foster City, CA, USA). Each 20-μL reaction consisted of 1 μL RT product; 10 μL SYBR Premix ExTaq™ enzyme (Takara, Dalian, China); 0.5 μL each upstream and downstream primers; and 8 μL ddH_2O. Reaction conditions were as follows: 95°C for 2 min; 44 cycles of 95°C for 10 s, 60°C for *NR5A2* or 68°C for *β-actin* for 20 s, and 72°C for 10 s; and 72°C for 5 min. Reactions were assessed by melting curves and were normalized against the housekeeping gene *β-actin*. Reactions were repeated in triplicate for each sample.

SNP screening and genotyping

We screened *NR5A2* coding sequences for SNPs using a DNA pooling sequencing assay with primers P1-3 (Table 1). Next, 5 μL100 ng/μL DNA was collected from the ten sheep and pooled. PCR products were sequenced in both directions. SNPs were identified using Chromas v2.31 (Technelysium Pty Ltd., South Brisbane Australia) and DNA star v7.0 software (DNASTAR, Inc. Madison, WI USA). SNPs were genotyped by restriction fragment length polymorphism (RFLP) or allele-specific (AS) PCR. For PCR-RFLP, 20 μL volumes contained 50 ng genomic DNA, 2 μL buffer, 1.4 μL Mg^{2+}, 0.5 μL each primer, and 0.2 μL Taq DNA polymerase. The cycling protocol was as follows: 5 min at 95°C; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 20 s; and a final cycle of 72°C for 10 min. PCR products (5 μL) using different primer pairs were mixed with 0.7 μL 10X buffer; 2.5 U restriction enzyme (*Mse*I); and 3.8 μL sterilized ddH_2O, and incubated for 3 h at

65°C. Digested products were subjected to 3% agarose (w/v) gel electrophoresis and genotypes were observed. For AS-PCR, specific primers were designed at SNP sites and included two forward and one reverse primer (Table 1). Bases at the 3′ ends of the two forward primers corresponded to the two SNP sequences, and the reverse primer was normally designed (Liu et al., 2012).

Statistical analysis

Allele frequencies, heterozygosity (H_E), and polymorphism information content (PIC) were calculated using PopGene v1.31 software (http://www.ualberta.ca/~fyeh/fyeh). Linkage disequilibrium was estimated using SHEsis software. Univariate analysis in the general linear model procedure of SPSS16.0 software was used to analyze the quantitative results and the association between various genotypes and litter size. Multiple comparisons of the means were performed using the least significant difference method, and the model applied was:

$$Y_{ikm} = \mu + G_i + P_k + E_{ikm}$$

In the above formula, Y_{ikm} is the trait measured on each of the ikm^{th} animal; μ is the average litter size of each animal; G_i is the fixed effect associated with *i*th genotype or combinative genotype; P_k is the fixed effect associated with parities; and E_{ikm} is the random error.

RESULTS

Identification and characterization of *NR5A2* of Hu sheep

The full-length coding region of Hu sheep *NR5A2* was 1488 bp, which was submitted to GenBank (accession No. JN182926). The coding region was 96.98% homologous to that of cattle, 88.37% homologous to humans, and 73.62% homologous to zebrafish at the nucleotide level. By blasting in NCBI, we found *Nr5a2* gene comprised at least seven exons and six introns, and was located at 76960-77100 kbp within NW_004080175.1 on chromosome 12 of sheep (*Ovis aries*).

The NR5A2 protein of Hu sheep contained 495 aa residues (Figure 1A); the relative molecular weight was 56.20 kDa and the isoelectric point 8.09. Using BLAST, we found that the Hu sheep NR5A2 protein was highly homologous at the aa level to that of cattle (98.38%) and humans (96.77%), but was only 76.98% homologous with that of zebrafish, indicating that NR5A2 is relatively conserved in mammalian species. We constructed a phylogenetic tree of mammals based on the aa sequences of NR5A2; clustering of genes on the tree was consistent with the traditional species classification (Figure 1B). Only 18 aa were represented in the NR5A2 protein sequence, of which Pro was the most abundant (11.27%); Pyl and Sec were absent. We detected 55 positively and 52 negatively charged aa residues. *In vitro* protein half-life was 30 h, and the instability coefficient was 50.24. The grand average of hydropathicity (GRAVY) was -0.362, suggesting that the NR5A2 protein was strongly hydrophilic. Our analysis did not predict a signal peptide, nor was NR5A2 predicted to be a secretory or transmembrane protein. The protein was mainly localized in the nucleus.

Further analysis indicated that sheep NR5A2 contained three conserved domains - a DNA-binding (DBD; Cys40 - Leu132); an Ftz-F1 box (Ala109-Ala138); and a ligand-binding (LBD) domain (Ala255-Ser495; Figure 1A). To further characterize the protein, we predicted its three-

dimensional structure using the SWISS-MODEL website online tool (http://swissmodel.expasy.org//SWISS-MODEL.html) according to the neural network homology modeling method and PyMol software for visualization, we found that NR5A2 contained 11 α-helices, 2 β-folds, and some random curls (Figure 1C).

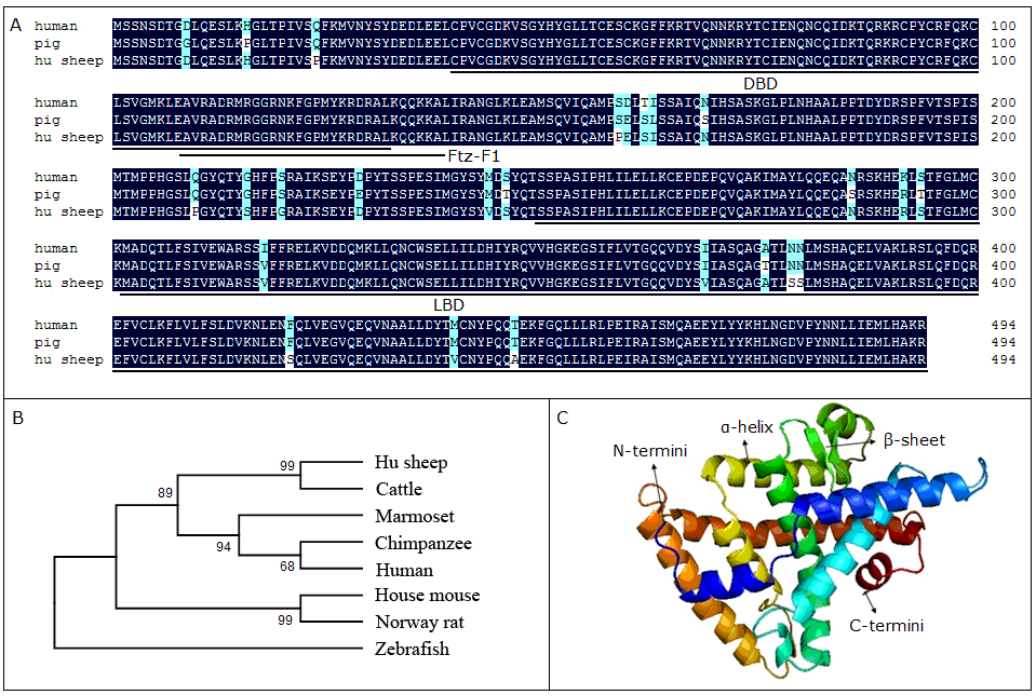

Figure 1. Identification of *NR5A2* in Hu sheep. Alignment of Hu sheep NR5A2 protein with human (NP_003813) and pig (AFD98842). Black boxes indicate positions at which residues are identical, and grey boxes highlight residues that are similar. Functional domains and motifs are underlined (**A**). A neighbor-joining tree of mammals was constructed based on the amino acid sequences of NR5A2 protein (**B**). 3-D structure of NR5A2 protein in Hu sheep (**C**).

NR5A2 mRNA level is significantly associated with reproductive performance

We evaluated the expression pattern of *NR5A2* in 11 tissue types from Hu sheep by RT-PCR, and found it was widely expressed in the heart, hypothalamus, liver, uterus, duodenum, fallopian, kidney, ovary, spleen, lung and muscle tissues, and was particularly highly expressed in the ovary (Figure 2A). To further investigate the relationship between NR5A2 and reproductive performance of Hu sheep, the mRNA level of NR5A2 in ovaries was also evaluated. There was a positive correlation between the *NR5A2* mRNA level in ovaries and ovulation rate (r = 0.8125; Figure 2B), and the litter size (r = 0.7092; Figure 2C).

NR5A2 contains two point mutations in Hu sheep

To search for SNPs in Hu sheep *NR5A2*, we used pooled DNA resequencing of the full coding region. Two SNPs, T40C and T1419C, were identified (Figure 3A and B). At the T40C locus,

we detected three genotypes (TT, CT, and CC) using the PCR-RFLP method (Figure 3C), and their genotype frequencies were 0.137, 0.751, and 0.111, respectively (Table 2). The allele frequencies C and T were 0.487 and 0.513, respectively. The PIC (Polymorphism information content) was 0.375. The T40C site was in Hardy-Weinberg disequilibrium ($P < 0.05$). We also detected three genotypes (TT, CT, and CC) at T1419C using AS-PCR (Figure 3D); the genotype frequencies were 0.115, 0.823, and 0.062, respectively (Table 2). The C and T allele frequencies were 0.473 and 0.527, respectively. The PIC was 0.374, and the site was in Hardy-Weinberg disequilibrium ($P < 0.01$).

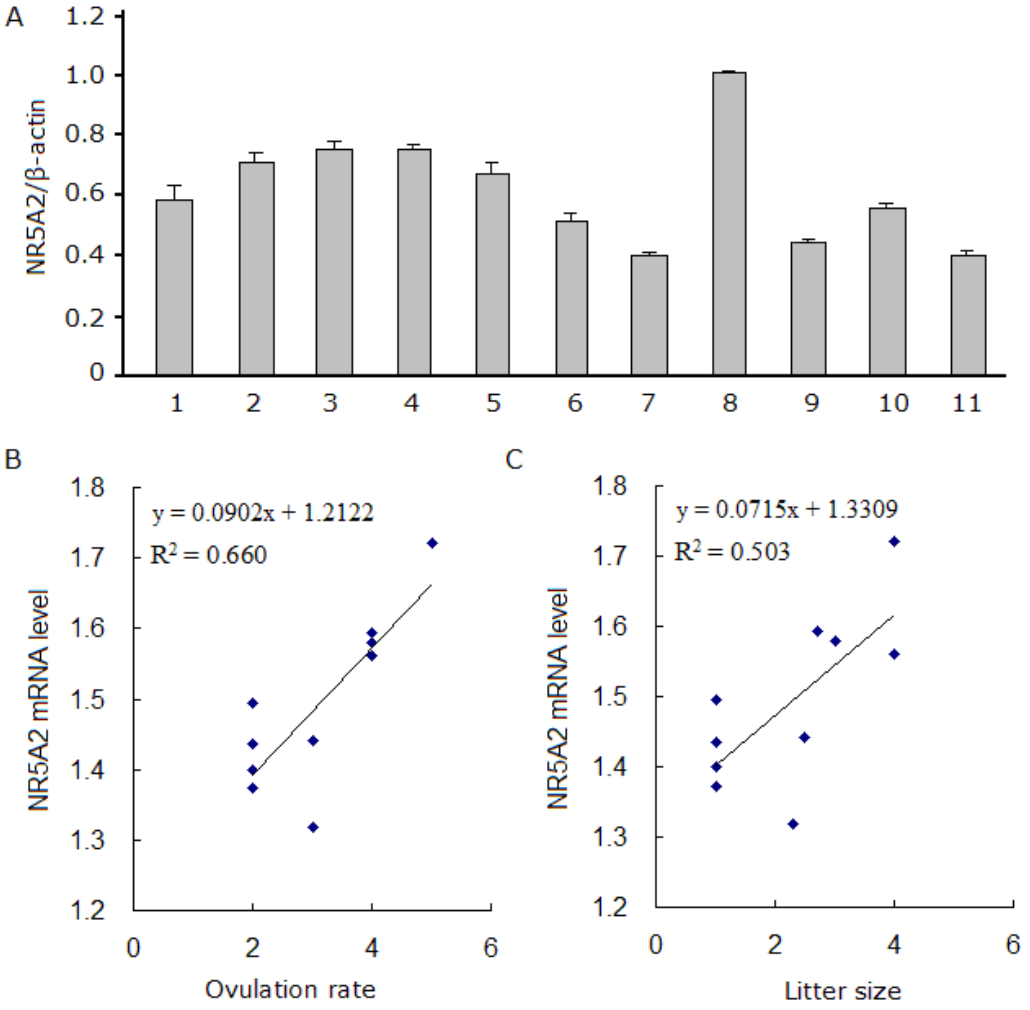

Figure 2. Relationship between *NR5A2* mRNA level and reproductive performance of Hu sheep. Tissue mRNA expression profiles of NR5A2, 1-11 indicate heart, hypothalamus, liver, uterus, duodenum, fallopian, kidney, ovary, spleen, lung and muscle samples (**A**). Relationship between NR5A2 mRNA level and ovulation rate (**B**) and between NR5A2 mRNA level and litter size (**C**).

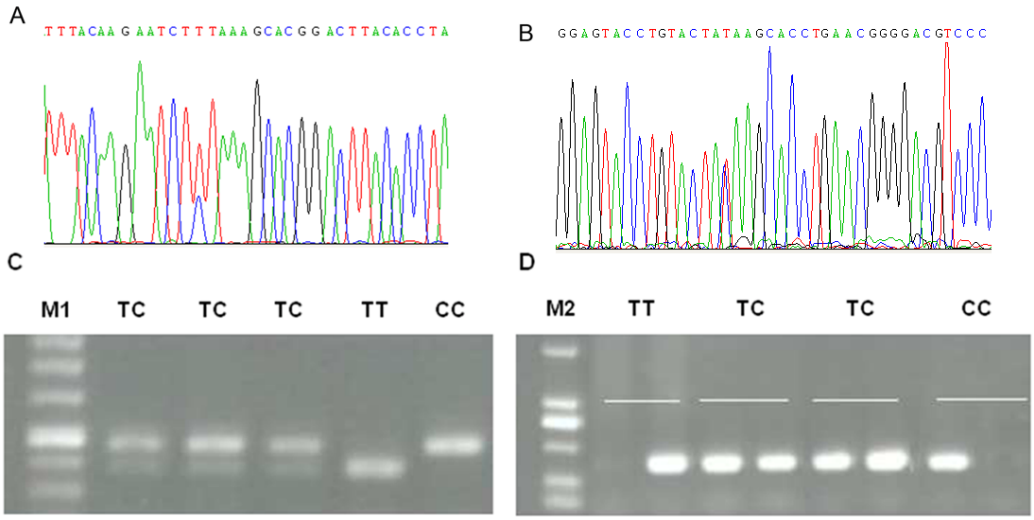

Figure 3. SNP identification and genotyping of *NR5A2* coding region. T40C (**A**) and T1419C (**B**) locus sequencing maps and genotype map enzyme by *Mse*I for T40C locus (**C**). AS-PCR electrophoresis map for T1419C locus (**D**).

Table 2. Genetic characteristics of two SNP loci in *NR5A2*.

SNP	Genotype frequency			Allele frequency		PIC	H_E
	CC	TC	TT	C	T		
T40C	0.110	0.755	0.135	0.487	0.513	0.375	0.500
T1419C	0.062	0.823	0.115	0.473	0.527	0.374	0.497

PIC = polymorphism information content; H_E = heterozygosity.

Association analysis of independent SNPs with litter size

The association between*NR5A2* polymorphisms with litter size in Hu sheep populations was verified. The litter size of Hu ewes with T40C genotype CC (1.77 ± 0.08) and TC (1.74 ± 0.07) were greater than that of ewes with genotype TT (1.49 ± 0.03; P < 0.05) at the average litter size. Ewes with genotype CC (2.50 ± 0.22) had larger litter sizes than those of ewes with genotype CT (1.72 ± 0.16) and TT (1.67 ± 0.08) at the third parity; ewes with genotype CT (1.81 ± 0.13) had larger litter sizes than those of ewes with genotype CC (1.67 ± 0.18) and TT (1.40 ± 0.05) at the second parity (Table 3). At the T1419C locus, individuals with the TT genotype had larger litter sizes than those with genotype CC or genotype CT, but the difference was significant only at the third parity (P < 0.05).

Table 3. Least squares mean for litter size of different genotypes of NR5A2.

Locus	Genotype	Number	1st parity litter size	2nd parity litter size	3rd parity litter size	Average litter size
T40C	CC	27	1.71 ± 0.12^{a}	1.67 ± 0.18^{ab}	2.50 ± 0.22^{a}	1.77 ± 0.08^{a}
	CT	179	1.69 ± 0.1^{a}	1.81 ± 0.13^{a}	1.72 ± 0.16^{b}	1.74 ± 0.07^{ab}
	TT	33	1.60 ± 0.05^{a}	1.40 ± 0.05^{b}	1.67 ± 0.08^{bc}	1.49 ± 0.03^{c}
T1419C	TT	27	1.73 ± 0.18^{a}	1.58 ± 0.18^{a}	2.00 ± 0.25^{a}	1.83 ± 0.14^{a}
	TC	197	1.68 ± 0.05^{a}	1.74 ± 0.07^{a}	1.73 ± 0.08^{ab}	1.67 ± 0.05^{a}
	CC	15	1.62 ± 0.17^{a}	1.67 ± 0.14^{a}	1.33 ± 0.26^{b}	1.58 ± 0.15^{a}

Values with different superscripts within the same column in particular locus differ significantly (P < 0.05).

DISCUSSION

NR5A2 is involved in a variety of processes including ovulation and embryo differentiation and development (Fayard et al., 2004; Hsieh et al., 2009; Yazawa et al., 2010; Winkler et al., 2010). Hinshelwood et al. (2005) found that NR5A2 plays a role in gonadal development, the initiation of folliculo genesis, and regulation of estrogen biosynthesis within the ovary. *NR5A2* has a great impact on animal reproductive performance (Hinshelwood et al., 2005; Duggavathi et al., 2008; Atkin et al., 2013). Duggavathi et al. (2008) found that female mice with granulosa cell-specific deletions of *NR5A2* are sterile, due to anovulation. Specific-knockout of *NR5A2* in ovarian corpus cells induces ovarian dysfunction and eventually leads to pregnancy failure (Zhang et al., 2013), and mice with hypothalamic arcuate nucleus with kisspeptin neuron-specific deletion of *NR5A2* have been found to have significantly reduced litter sizes (Atkin et al., 2013). In this study, we cloned and characterized the full-length coding sequence of *NR5A2* of Hu sheep. We detected high homology between Hu sheep and other mammals for both the nucleotide and aa sequences, suggesting conservation of mammalian *NR5A2* during evolution. Furthermore, like other mammals, Hu sheep NR5A2 has three classic conserved functional domains - DBD, Ftz-F1 box, and LBD (Li et al., 1998; Fayard et al., 2004). For members of the NR5A family, specific binding to DNA is determined by the Ftz-F1 box domain, a 26-aa region adjacent to the C-terminus of the DBD domain (Ueda et al., 1992). Together, these results indicate that *NR5A2* is highly conserved in mammals, and that Hu sheep NR5A2 may contribute to female reproduction and reproductive development, as is the case for other mammals (Labelle-Dumains et al., 2007; Guo et al., 2010).

In mammals, *NR5A2* is expressed mainly in the liver, intestine, pancreas and ovary (Falender et al., 2003), and has been detected in other tissues such as the hypothalamus and duodenum, indicating that it may play an important role in other tissues and organs (Baquié et al., 2011). In the current study, we found that *NR5A2* was widely expressed in various tissues and was highly expressed in the ovary, consistent with previous reports (Falender et al., 2003). We found a positive correlation between *NR5A2* mRNAs level in the ovary and ovulation rate and litter size, suggesting that *NR5A2* was involved in regulating reproductive performance. NR5A2 knockout mice died at day 6.5-7.5 of gestation, and the fecundity of heterozygous mice decreased significantly (Labelle-Dumains et al., 2007). Mice lacking NR5A2 in granulosa cells were sterile owning to anovulation (Duggavathi et al., 2008). Together, these data suggest that *NR5A2* plays an important role in animal ovulation and reproduction (Labelle-Dumains et al., 2007; Duggavathi et al., 2008; Wang et al., 2013).

Hu sheep are a famous, highly prolific breed in China, and the molecular mechanism of its high fecundity has been a topic of interest for domestic scholars. At present, *IGF1* (He et al., 2012), *FSHR* (Chu et al., 2012a), *KISS-1/GPR54* (Chu et al., 2012b), *PPNR* (Guan et al., 2011), *INHBB* (Chu et al., 2011a) and *BMPR-IB* (Chu et al., 2011b) have been identified as candidate genes affecting Hu sheep fecundity. However, until now, no gene with a major effect on reproduction has been identified. *NR5A2* research has mainly focused on correlations between SNPs and pancreatic cancer (Rizzato et al., 2011; Tang et al., 2014), and no studies have been reported that examine the relationship between *NR5A2* SNPs and reproduction in domestic animals. We identified two SNPs - T40C and T1419C - in the coding region of Hu sheep *NR5A2*, and found that the T40C locus was indeed correlated with litter size. We also found that the T1419C locus was located in the LBD region, furthermore, like the T40C locus, this polymorphism change the aa from Leu to Ile, and the molecular mechanism underlying the effect on litter size of Hu sheep needs to further research.

In the present study, we found the full coding sequence of *NR5A2* was 1488 bp and was conserved in mammals. There was a positive correlation between the mRNA level of *NR5A2* in ovary and ovulation rate and litter size. T40C and T1419C loci were detected in the coding sequence of *NR5A2*. Association analysis showed that the T40C SNP could be used as a genetic marker for selecting individuals with higher litter sizes in Hu sheep.

Conflicts of interest

The authors declare no conflicts of interest.

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