A T > G Mutation in the NR5A2 Gene Is Associated With Litter Size in Hu Sheep Through Upregulation of Promoter Activity by Transcription Factor MTF-1

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Nuclear receptor subfamily 5 group A member 2 (NR5A2), also referred to as LRH-1 or FTF, is an orphan nuclear hormone receptor that is involved in regulating embryonic development, ovarian granulosa cell differentiation, gonadal sex differentiation, and steroidogenesis in mammals. However, little is known about how NR5A2 regulates reproduction in sheep. In this study, we amplified the promoter sequence of NR5A2 and determined that its core promoter region ranged from -721 nt to -281 nt. A T > G polymorphism at -700 nt was detected in the core promoter region. Association analysis found that the litter sizes of Hu ewes at their second and average parities with genotype GG (2.20 ± 0.20 and 1.97 ± 0.06, respectively) were significantly higher than those of ewes with genotype TG (1.68 ± 0.10 and 1.74 ± 0.05, respectively) (p < 0.05) and TT (1.67 ± 0.10 and 1.62 ± 0.06, respectively) (p < 0.05). The litter size of Hu ewes at their third parity with genotype GG (2.10 ± 0.10) was significantly higher than that of ewes with genotype TT (1.56 ± 0.12) (p < 0.05). A luciferase assay showed that the -700G allele increased the luciferase activity relative to the -700T allele. Furthermore, the -700T > G polymorphism created a novel binding site for metal-regulatory transcription factor 1 (MTF-1). A competitive electrophoretic mobility shift assay confirmed that MTF-1 specifically bound with the G-type promoter of NR5A2. An overexpression experiment demonstrated that MTF-1 was involved in the alteration of NR5A2 transcription activity and further increased NR5A2 gene mRNA expression. Our findings revealed that the -700T > G polymorphism promoted NR5A2 expression due to the positive effects on NR5A2 gene transcription activity by MTF-1 and thereby increased fecundity in Hu sheep.

Keywords: Hu sheep, NR5A2, core promoter, single nucleotide polymorphism, competitive EMSA, MTF-1

INTRODUCTION

Nuclear receptor subfamily 5 group A member 2 (NR5A2), also referred to as liver receptor homolog-1 (LRH-1) or fetoprotein transcription factor (FTF) in Drosophila (Galarneau et al., 1996), is an orphan nuclear hormone receptor. It is widely expressed in all tissues, especially in the ovaries (Falender et al., 2003; Li et al., 2015), and is involved in embryonic development (Wei et al., 2018),...
ovarian granulosa cell differentiation (Meinsohn et al., 2017), gonadal sex differentiation (Yan et al., 2018), and steroidogenesis (Camats et al., 2015; Xiao et al., 2018).

Meinsohn et al. (2017) found that NR5A2 played a role in granulosa proliferation and follicle growth. The fecundity of heterozygous mice (NR5A2+/−) significantly decreased (Duggavath et al., 2008). Mice lacking NR5A2 in granulosa cells were sterile as a result of anovulation. Kisspeptin neuron-specific NR5A2 knockout mice were shown to have significantly reduced litter sizes (Atkin et al., 2013). Polymorphisms in the NR5A2 gene were associated with preterm birth in humans (Dudai et al., 2016) and litter size in Hu sheep. The mRNA level of the NR5A2 gene is significantly associated with the ovulation rate and litter size of Hu sheep. This suggests that the NR5A2 gene plays an important role in regulating the reproductive performance of female animals.

Our previous study demonstrated that polymorphism in the promoter region of the ovine NR5A1 gene, another member of the NR5A family, is associated with litter size (Li et al., 2018). However, the polymorphism of the NR5A2 promoter region and its relationship with litter size in Hu sheep is still unknown. In this study, we examined the 5’ regulatory region of the NR5A2 gene, identified its core promoter region, screened SNPs in the core promoter region, determined the relationship of this gene with litter size in Hu sheep, and further explored the potential molecular mechanism of the NR5A2 gene. All these data will be important in elucidating the molecular characteristics of NR5A2, understanding its function in regulating the litter size in Hu sheep, and comprehensively exploring the molecular mechanism of the NR5A family that is involved in regulating the high fecundity of sheep.

MATERIALS AND METHODS

Samples

Ear tissue was collected from 193 Hu ewes (they are independent individuals and there is no relationship among them) from Helen Sheep Industry Co., Ltd., Jiangsu Province, China. DNA was extracted using a standard phenol–chloroform extraction protocol (Psifidi et al., 2010). Five healthy Hu ewes were chosen from the Dongshankou sheep conservation field in Jiangsu Province. After euthanization, eight tissue samples were collected (ovary, uterus, heart, liver, spleen, kidney, lung, and muscle tissue) and immediately placed in liquid nitrogen before being stored at −80°C. RNA was extracted from tissue samples using RNA extract kits (Bioteke, China). All experiments were performed in accordance with the protocol approved by the Committee on Ethics of Animal Experimentation from the Jiangsu Academy of Agricultural Sciences (No. 63 of Jiangsu Academy of Agricultural Sciences, approved on 8 July 2014).

Cloning, Sequencing, and Genotyping

A PCR was conducted using a 20-μl mixture containing 1 μl of reverse transcription (RT) product or 60 ng of DNA, 2 U of Taq DNA polymerase (Takara, Japan), 2 μl of 10× PCR buffer, 0.5 mmol/L dNTPs, 2.5 mmol/L MgCl₂, and 0.5 μl of 10 nmol/L upstream and downstream primers (Table 1). Amplification conditions were as follows: 94°C for 4 min; 94°C for 30 s, annealing temperature (listed in Table 1) for 30 s, and 72°C for 30 s for 35 cycles; and 72°C for 5 min. DNA purification and sequencing methods followed those of Li et al. (2015).

NR5A2 promoter sequences were screened for SNPs using a DNA pooling sequencing assay with primers P4 including core promoter region of NR5A2 gene (Table 1). Five microliters of 100 ng/μl DNA was collected from five ewes and pooled

| TABLE 1 | Primers used in this study. |
|---|---|
| **Primers** | **Primers sequence (5′-3′)** | **Tm(°C)** | **Size(bp)** | **Usage** |
| P1 | F: ATAAGCTGAACAATCTCTGATATTGAAG | 56 | 265 | constructed pGL3-281 vector of NR5A2 gene |
| P2 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 56 | 706 | constructed pGL3-721 vector and pGL3-G of NR5A2 gene |
| P3 | F: ATAAGCTGAACAATCTCTGATATTGAAG | 56 | 1357 | constructed pGL3-1373 vector of NR5A2 gene |
| P4 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 55 | 575 | SNP screening of NR5A2 promoter |
| P5 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 56 | 706 | Constructed pGL3-T vector of NR5A2 gene |
| P6 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 56 | 195 | primers of NR5A2 for real-time PCR |
| P7 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 68 | 113 | primers of β-actin for real-time PCR |
| P8 | R: ATGACCGGATCCGATCTTCCTTGGTTAG | 55 | 2462 | mtf-1 overexpression |
| P9 | R: AGAAGCAACCTGGCACTGAGCCAA | 56 | 0 | Bi-probe |
| P10 | R: AGAAGCAACCTGGCACTGAGCCAA | 56 | 0 | Mut-probe |
| P11 | R: AGAAGCAACCTGGCACTGAGCCAA | 56 | 0 | cold probe |

**Underline indicates restriction enzyme sites**
together. PCRs were sequenced in both directions. SNPs were identified using Chromas v2.32 (Technelysium, Australia). SNPs of 193 ewes were genotyped with PCR-RFLP using Hin6I endonuclease (Takara, Japan) at 37°C for 3 h.

Bioinformatic Analysis

The NR5A2 promoter sequence was analyzed using DNASTAR (DNASTAR Inc., Madison, WI). The core promoter was predicted using Promoter 2.0 (Knudsen, 1999) and transcription binding sites in the promoter were predicted using JASPAR (http://jaspar.binf.ku.dk/).

Construction of Plasmids

To identify the core promoter region, three NR5A2 promoter deletion fragments (1: -281 to -17 bp; 2: -721 to -17 bp; 3: -1373 to -17 bp; these three fragments were only different in length but did not include deletions of different functional elements) were amplified and cloned in the Nhel–Xhol (Takara, Japan) site of a pGL3-basic vector to create pGL3-281, pGL3-721, and pGL3-1373 products. The MTF1 CDS sequence of Hu sheep was amplified and cloned in the Xhol–XbaI (Takara, Japan) site of a pGL3-basic vector (Fig. 1). Synthetic oligonucleotides 5′-labeled with biotin (Invitrogen, USA) were annealed to generate double-stranded oligonucleotides as probes (Table 1). The bio-probe is a wild-type probe labeled with biotin at the 5′ end; the cold probe, also called the competitive probe, is unlabeled and has the same sequence as that of labeled probes; the mut-probe is also unlabeled, and has the sequence as that of labeled probes, but the binding domain has been mutated with two bases. EMSAs were performed according to the instructions provided in the Competitive EMSA Kit (Viagene Biotech, Changzhou, China). Briefly, 3 µg of nuclear extract was incubated at 20°C for 20 min in the presence of 1.5 µl 10× binding buffer, 0.25 µl Poly (dl-dC) in a reaction volume of 14.5 µl, and then 0.5 µl of bio-probe was added, mixed well, and let the mixture sit at 20°C for 20 min. For competition experiments, unlabeled wild-type oligonucleotides (cold probe) and unlabeled mutant-type oligonucleotides (mut-probe) were added in a 100× molar excess prior to the addition of the biotinylated probe. Samples were electrophoresed through a 4% non-denaturing polyacrylamide gel in 0.5× Tris/Borate/EDTA at 4°C until the bromophenol blue reached the lower end of the gels (about 50–80 min), then electrotransferred and cross-linked by ultraviolett. Chemiluminescence imaging showed bands of DNA binding to transcription factors.

Statistical Analyses

The allele frequencies, heterozygosity, and polymorphism information content were calculated using PopGene v1.31 software (http://www.ualberta.ca/~fyeh/fyeh). Linkage analyses among different loci were performed with Haploview 4.2 (Barrett et al., 2005). The association analysis between different genotypes and the litter size of Hu sheep was referenced by Li et al. (2015). All results are reported as mean values ± SEM. Statistical analysis was performed with independent sample t-tests using SPSS 16.0 statistical software (SPSS Inc., USA).

RESULTS

Identification of the Core Promoter Region of the Ovine NR5A2 Gene

To identify the core promoter region of NR5A2 gene, three deletion plasmids, pGL3-281, pGL3-721, and pGL3-1373 (Figure 1), were
transfected into 293T cells. The luciferase assay showed that the luciferase activity of plasmid pGL3-721 was significantly higher than that of pGL3-basic and pGL3-281 (p < 0.01). However, the difference in luciferase activity was not statistically significant between pGL3-721 and pGL3-1373 (p > 0.05), which revealed that the location of the core promoter region of the ovine NR5A2 gene ranged from -721 to -281 bp (Figure 1). Additionally, many binding sites for transcription factors, such as C/EBPα, NF-Y, c-FOS, AP3, GATA2, SBF1, USF2, and MTF-1, were predicted to be present in this region (Supplementary Figure 1).

SNP Identification in the Core Promoter Region and Correlation With Litter Size in Hu Sheep

SNPs in the NR5A2 gene promoter region were explored by DNA pool sequencing, and a T/G mutation was identified at -700 nt in the core promoter region (Supplementary Figure 2A). Genotyping showed that GG, GT, and TT genotypes were found in 192 Hu ewes (Supplementary Figure 2B), with genotype frequencies of 0.104, 0.563, and 0.333, respectively. Association analysis showed that the litter sizes of Hu ewes at their second and average parities with genotype GG (2.20 ± 0.20 and 1.97 ± 0.06, respectively) were significantly higher than those of ewes with genotype TT (1.56 ± 0.12; p < 0.05), and there was no significant difference between the litter sizes of ewes with genotypes GG (2.10 ± 0.10) and TG (1.85 ± 0.10; p > 0.05) (Table 2). These results indicate that the -700T/G polymorphism in the core promoter region of NR5A2 gene was involved in regulating the litter size of Hu sheep.

The -700T/G Polymorphism Affected Promoter Activity of the NR5A2 Gene

To investigate whether the -700T/G polymorphism affected promoter activity of the NR5A2 gene, constructs of pGL3-T and pGL3-G were transfected into 293T cells, and the results showed that luciferase activity of plasmid pGL3-G was significantly higher than that of plasmid pGL3-T in 293T cells (p < 0.05) (Figure 2).

The -700T/G Polymorphism Adds a Novel Transcription Factor Binding Site

We predicted that the -700T/G polymorphism would create a novel binding site for transcription factor MTF-1. A competitive EMSA showed that when nuclear extracts of ewes’ ovaries were incubated with a bio-probe, there was a significant DNA–protein complex, and when nuclear extracts were incubated with a bio-probe and mut-probe at the same time, the DNA–protein complex was reduced (Figure 2).

| genotype | number | 1st parity litter size | 2nd parity litter size | 3rd parity litter size | average litter size |
|----------|--------|------------------------|------------------------|------------------------|---------------------|
| GG       | 20     | 1.80 ± 0.13a           | 2.20 ± 0.20a           | 2.10 ± 0.10a           | 1.97 ± 0.06a        |
| GT       | 108    | 1.63 ± 0.07a           | 1.68 ± 0.10b           | 1.85 ± 0.10ab          | 1.74 ± 0.05b        |
| TT       | 64     | 1.56 ± 0.12a           | 1.67 ± 0.10bc          | 1.56 ± 0.12c           | 1.62 ± 0.06bc       |

Values with different superscripts within the same column in particular locus differ significantly at P < 0.05.
complex was still present. However, when nuclear extracts were incubated with a bio-probe and cold probe at the same time, DNA–protein complex significantly decreased with the cold-probe concentration increased. These results suggest that MTF-1 binds specifically to sequences with -700GG (Figure 3).

**MTF-1 Was Involved in Regulating NR5A2 Gene Expression**

To determine whether MTF-1 was involved in regulating NR5A2 gene expression, the plasmid pcDNA3.1-MTF-1 was transfected in sheep granulosa cells. The qRT-PCR results showed that the MTF-1 expression levels were higher in sheep granulosa cells transfected with plasmid pcDNA3.1-MTF-1 ($p < 0.01$) (Figure 4), and expression levels of the NR5A2 gene increased after pcDNA3.1-MTF-1 transfection (Figure 4). Additionally, qRT-PCR results showed that MTF-1 was widely expressed in Hu sheep tissues, particularly in the ovary and uterus (Figure 4). These data suggest that MTF-1 was involved in regulating NR5A2 gene expression in the ovarian granulosa cells of Hu sheep.

**-700T/G Polymorphism Affected MTF-1 Regulation of NR5A2 Promoter Activity**

$p$cdNA3.1-MTF-1 was co-transfected with pGL3-T or pGL3-G into 293T cells. The promoter activity assay showed that the overexpression of MTF-1 significantly enhanced the luciferase activity of the -700G promoter genotype ($p < 0.01$), but the -700T promoter genotype did not have a significant effect on luciferase activity ($p > 0.05$) (Figure 2).

**DISCUSSION**

NR5A2 is a transcription factor involved in regulating metabolism and hormone synthesis (Cobo-Vuilleumier et al., 2018; Jiang et al., 2018). Studies have shown that NR5A2 is essential for pregnancy and reproduction in female animals (Brosens et al., 2013; Zhang et al., 2013; Guo et al., 2016; Kaluarachchi et al., 2016). A previous study showed that the expression level of NR5A2 was associated with ovulation and litter size in Hu sheep (Li et al., 2015). In this study, we focused on the 5′ regulatory region of NR5A2 and determined that its core promoter region ranged from -721 to -182 nt. A -700T/G polymorphism was detected at the core promoter region of the NR5A2 gene. Polymorphisms of NR5A2 are associated with preterm birth in humans (Kaluarachchi et al., 2016), and it has been shown that T40C and T1419C at the NR5A2 CDS region are associated with litter size in Hu sheep (Li et al., 2015). In this study, we found that -700T/G located in the core promoter of NR5A2 was significantly associated with litter size in Hu sheep, with ewes with genotype GG having a significantly greater litter size at the second and average parities than those with genotypes...
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TG (p < 0.05) and TT (p < 0.05). Moreover, ewes with genotype GG had a significantly greater litter size at the third parity than those with genotype TT (p < 0.05). By combining all three polymorphisms (-700T/G, T40C, and T1419C) of the NR5A2 gene, we found that ewes with the GG/TC/TC haplotype have higher litter sizes in the second and third parities than those with other haplotypes (Supplementary Table 1), suggesting that the NR5A2 gene could be used as a candidate molecular marker for breeding high-fertility sheep.

We found that the T/G polymorphism changed the promoter activity of the NR5A2 gene, suggesting that this mutation was probably involved in regulating NR5A2 gene expression, affecting the litter size of sheep as a result. Further analysis showed that -700G created a transcription factor MTF-1 binding site and MTF-1 was involved in regulating NR5A2 promoter transcription activity, increasing the NR5A2 gene expression in granulosa cells of sheep. Xu et al. (2018) found that polymorphisms of the HOMER1 gene are associated with piglet splay leg syndrome and the G allele of rs325197091 (A > G) may create a new binding site of transcription factor ARNT, which could enhance HOMER1 promoter activity and expression. Promoter haplotypes of the ABCB1 gene encoding the P-glycoprotein differentially affects its promoter activity by altering the transcription factor binding (Speidel et al., 2018).

**FIGURE 4** | MTF-1 involved in regulating NR5A2 gene expression in ovine ovarian granulosa cells. (A) MTF-1 gene expression after transfection in pcDNA3.1-MTF-1 in ovine ovarian granulosa cells. (B) Overexpression of MTF-1 increases the NR5A2 gene expression in ovine ovarian granulosa cells. (C) MTF-1 expression profiles in ovine tissues. **p < 0.01.

**FIGURE 5** | Flow diagram of the -700T/G mutation of NR5A2 regulating ovine reproduction through MTF-1. -700T/G polymorphism of NR5A2 gene core promoter creates a novel transcription factor MTF-1 binding site, upregulates the transcription activity and mRNA expression of NR5A2 gene in granulosa cells, and then increases the litter size of Hu sheep probably due to regulating steroidogenesis of NR5A2 and further affecting ovulation and litter size.
MTF-1, a six-zinc-finger protein of the Cys2His2 family, binds directly and specifically to metal response elements, and regulates metal-induced and basal MT expression (Heuchel et al., 1994; Radtke et al., 1995). As a transcription activator, MTF-1 plays different roles and is involved in metal and oxidative stress (Dalton et al., 1996; Ben Mimouna et al., 2018) and embryological development (Hogstrand et al., 2008). Nishimoto et al. (2009) found that MTF-1, as a transcription activator, binds with the promoter region of PIGF, increases its expression, and is involved in the hypoxia-dependent regulation of PIGF in trophoblast-derived cells. MTF-1 could also be a transcription factor that binds to FSH, CYP19A2, and 20β-HSD, and is also involved in regulating steroidogenesis synthesis in zebrafish (Urbatzka et al., 2012). In this study, we found that MTF-1 is a transcription activator that can bind with an NR5A2 -700GG type promoter, increase its promoter activity and expression level in granulosa cells, and further regulate the ovulation and litter size of Hu sheep (Figure 5).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All experiments were performed in accordance with the protocol approved by the Committee on Ethics of Animal Experimentation from the Jiangsu Academy of Agricultural Sciences (No. 63 of Jiangsu Academy of Agricultural Sciences, approved on 8 July 2014).

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AUTHOR CONTRIBUTIONS

YL is responsible for the design of the whole article. JZa is responsible for SNP screening. YQ is responsible for samples collection. CM is responsible for real-time PCR. HW is responsible for cell assay. SC is responsible for EMSA assay. JZo is responsible for the modification of the whole article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01011/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Characterization of the NR5A2 promoter region in Hu sheep.

SUPPLEMENTARY FIGURE 2 | SNP identification and genotyping of the -700T/G mutation in the ovine NR5A2 gene.

SUPPLEMENTARY TABLE 1 | The association between different haplotype and litter sizes in Hu sheep.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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