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Role of FGF9 in sheep testis steroidogenesis during sexual maturation

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ABSTRACT

Fibroblast growth factor 9 (FGF9) is an important signaling molecule in early gonadal development. Hu sheep are noted for reproductive precociousness and fertility. The present study was conducted to investigate the gene expression and functions of FGF9 in ovine testis steroidogenesis during sexual maturity. A 874 bp cDNA fragment of *FGF9* was detected that included a 627 bp coding sequence, encoding 208 amino acids. The FGF9 amino acid sequence of sheep had high homology with this molecule of other mammalian species. Additionally, the abundance of FGF9 in ovine testis was greater ($P < 0.05$) at 9 months (M) and 24 M of age compared with those at 3 M. Immunohistochemistry further revealed that FGF9 mainly localized in the Leydig cells and that there were small amounts in elongating spermatids. The functions of FGF9 in sheep Leydig cells was investigated using a siRNA-FGF9. Secretion of T and abundance of testosterone synthesis-related enzymes in Leydig cells were inhibited ($P < 0.05$) by siRNA-FGF9. Thus, these results demonstrated FGF9 is an important regulator of testosterone biosynthesis in rams. Results of the present research provide a new perspective for genetic and molecular research on modulation of physiological mechanisms during sexual maturity in male sheep.

1. Introduction

Fibroblast growth factor 9 (FGF9) is a member of the FGF family, that was first isolated from human glioma cells (Naruo et al., 1993). Recently, a number of studies have revealed that FGF9 is associated with lens fiber differentiation, neuron development, bone formation, and gap junction formation (Lovicu and Overbeek, 1998; Cohen and Chandross, 2000; Schreiber et al., 2012). Furthermore, previous reports have demonstrated the role of FGF9 in sex determination during fetal development (Colvin et al., 2001a). Meiosis of germ cells is directly inhibited by FGF9 in fetal testis, which identified the critical and direct role of FGF9 in germ cell sex determination (Bowles et al., 2010). Furthermore, FGF9 has an important role in regulating female rat ovarian function and male mouse gonadal development (Drummond et al., 2007; Hiramatsu et al., 2009, 2010; Chung et al., 2013). As observed in mice and human embryos, the FGF9 gene, as a candidate gene for being a testis-determining factor, is expressed in germ cells following testis differentiation which suggests an important role for this molecule in early gonadal development and germ cell maturation (Ostrer et al., 2008). In postnatal stages, the expression of FGF9 gene was greatest in the mouse Leydig cell, spermatids, and spermatocytes. Furthermore, the FGF receptor gene for FGFR2 was extensively expressed in Leydig cells and spermatids, and the FGFR3 gene was widely expressed in the whole mouse testis. The relative expression of the FGF9 gene correlates with the temporal expression profiles

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of the FGF receptors FGFR2 and FGFR3, which suggests that FGF9 is associated with testis development (Lai et al., 2016). In addition, previous research indicated that the FGF9 gene was expressed in mouse Leydig cells, and that FGF9 stimulates testosterone synthesis in a dose- and time-dependent manner. (Lin et al., 2010; Lai et al., 2014).

The sheep is an excellent model for experimental physiological studies due to its large size and temperament. Furthermore, Hu sheep is a Chinese indigenous breed with precociousness and high fertility. The aim of the present study was to investigate the function of FGF9 in the sheep testis during sexual maturity. In the present study, the ram *FGF9* sequence was cloned and further investigated expression of the FGF9 gene was assessed in the testis of rams during sexual maturity. Additionally, the role of FGF9 in testosterone synthesis was also investigated in purified ovine Leydig cells. The present study provides a novel perspective for genetic and molecular studies on testis steroidogenesis in sheep during sexual maturation.

2. Material and methods

The study was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (SYXK2011-0036) and National Institutes of Health Guide for Care and Use animals. The methods were conducted in accordance with the approved guidelines.

2.1. Preparation of animals and tissues

Fifteen healthy Hu male sheep were selected according to birth records, and divided into three groups according to the various developmental stages, including prepuberty (3 months; $n = 5$), sexual maturity (9 M; $n = 5$), and adult (24 M; $n = 5$). Blood samples (15 mL) from each animal were obtained via jugular vein venipuncture, centrifuged at 1500 $\times g$ for 10 min at 4 °C, the serum was obtained and stored at −20 °C for enzyme-linked immunosorbent assays (ELISAs). Animals after assessment to be in a healthy physiological stage were deeply anesthetized by intravenous administration of 3% pentobarbital sodium, and sacrificed by exsanguination (Gao et al., 2017). Testis tissues were surgically removed and separated. One testis was immediately frozen in liquid nitrogen, and stored at −80 °C until RNA and protein extraction. The other was fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h, and embedded in paraffin for immunohistochemistry.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol reagent according to the method provided by the manufacturer (Invitrogen, Carlsbad, CA, USA). The concentration and quality of the RNA were detected using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples that had a 260/280 OD value between 1.8 and 2.0 were selected for further analysis. Reverse transcription reagent kits (Takara, Dalian, China) were used, and 1 μg of RNA in a final volume of 20 μL was then transcribed into cDNA (Guo et al., 2017). The cDNA was then stored at −20 °C until qRT-PCR analysis.

2.3. Cloning of FGF9

To obtain the coding sequence of FGF9 in rams, specific primers of FGF9-cDNA were designed using Primer 5.0 software, and evaluated using BLAST at NCBI (Table 1). The ram testis cDNA was amplified by PCR under the special conditions: 95 °C for 3 min, 35 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 60 s and 72 °C for 7 min. The PCR product was separated using 1.2% agarosegel. The purified PCR products in the right size were cloned into pClone 007 Blunt Vector (Tsingke, Beijing, China) and then transformed into *Escherichia coli* DH5a cells. Positive clones were selected randomly, and sequenced by Tsingke Biological Technology (Beijing, China).

2.4. Quantitative real-time PCR

Primers were designed online using Primer 5 software and evaluated using BLAST searching at NCBI. The qRT-PCR was performed using SYBR green (Roche, Germany) in a reaction volume of 20 μL, and according to a previously described protocol (Guo et al., 2017). Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (Ct; cycle threshold). The expression data were normalized to that of a house-keeping gene (*ACTB*), and the primer sequences are listed in Table 1.

Table 1
Primers and sizes of the amplification products of the target and housekeeping genes.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'	Product size, (bp)
FGF9-CDs	GACTATCCGCGGTTTGACCT	GGGCTCAAGTGAAGAAACGG	874
FGF9	CGTGGACAGTGGACTCTAC	TGCTTATACAGGTTGGAGGAG	138
StAR	GGGCATCCTCAAAGACCAG	TCCACCACCACCTCCAAC	120
3β-HSD	ATCCACACCAGCACCATAG	TTCCAGCACAGCCTTCTC	144
CYP17A1	GCCAACGTGCTGTGTGATTT	TTCGCGTTTCAACACAACCC	271
ACTB	CCAAGGCCAACCGTGAGAAG	CCATCTCCTGCTTCGAAGTCC	349