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Relative abundance of G protein-coupled receptor 30 and localization in testis and epididymis of sheep at different developmental stages

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ABSTRACT

The G protein-coupled receptor 30 (GPR30) is a transmembrane estrogen receptor that binds to estrogen, and has been confirmed to have an important role in testicular cell proliferation and development. The main objective of this study was to examine GPR30 gene expression and localization in the testis and epididymis of sheep at different developmental stages. Testes, including the epididymis, were collected from Polled Dorset x Mongolian cross rams at one ($n = 4$; wt), three ($n = 4$; wt), six ($n = 4$; wt), nine ($n = 4$; wt) and 12 ($n = 4$; wt) months of age. The 12-month-old hybrid crossbred sheep were exsanguinated *via* puncture of the jugular vein. Relative abundance of GPR30 mRNA was detected by quantitative PCR, and localization of the protein was examined by immunohistochemistry. Semi-quantitative analysis of GPR30 protein was performed by western blotting. The relative abundance of GPR30 mRNA was similar in the epididymis tail for rams at 6, 9, and 12mo of age. Further, relative abundance of GPR30 mRNA in the testes and caput epididymis of 1-, 3-, 6-, 9-, and 12-month-old crossbred rams did not increase with age. The GPR30 mRNA was detected in epididymal interstitial and principal cells, and in the epididymal cavity, spermatocytes, spermatogonial stem cells, Sertoli and Leydig cells, and spermatozoon of ram testes. Western blotting indicated the GPR30 protein was present in 9- and 12-month-old crossbred sheep corpus, cauda epididymis ($P < 0.05$). The results suggest that relative abundance of GPR30 mRNA is time-dependent and localization-specific.

1. Introduction

The G protein-coupled receptor 30 (GPR30) is a transmembrane estrogen receptor that binds to estrogen; GPR 30 has been suggested as being involved in rapid responses to estrogens in different cells, and thus has an important regulatory role (Wong et al., 2002; Watson et al., 2007; He et al., 2009). Chieffi (2015) assessed the role of GPR30 in testicles, and confirmed its importance in testicular cell proliferation and development (Chieffi, 2015). In this context, studies have focused on searching for sites of gene expression and specific location within reproductive organs. Immunohistochemistry techniques have been used to locate GPR30 in human Leydig and spermatogenic cells (Otto et al., 2009; Franco et al., 2011; Rago et al., 2011), while Chevalier et al. (2012) used gene expression technologies to analyze the expression of the GPR30 gene in

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normal human Sertoli cells, spermatogonia, and spermatocytes. Other studies have described the presence of GPR30 mRNA with *in vitro* studies of cultured human spermatocytes (Alves et al., 2012a,b, 2013). These findings were further confirmed with *in vitro* culture of testicular cells and spermatocytes (Oliveira et al., 2014). In this previous study, it was also demonstrated the GPR30 mRNA and protein were present in spermatocytes, Sertoli cells, and spermatozoon.

Most previous studies of GPR30 have focused on human testicular cells, and to the best of our knowledge, no study has examined the presence of mRNA and localization of GPR30 protein in the epididymis of sheep, or in other production animals. Thus, the general objective of the present study was to determine whether GPR30 mRNA and protein were present in the epididymis and testes of crossbred rams at different developmental stages.

2. Material and methods

2.1. Experimental animals and study design

2.1.1. Experimental animals

There were 20 healthy crossbred used in the present study (divided into five different age groups, each age group has four ram). Rams were obtained from the cross of a Polled Dorset sheep (♂) × Mongolian sheep, provided by the Breeding Center of Feed and Veterinary Medicine Research Institute, Shanxi Academy of Agricultural Sciences. Testes including the epididymis was collected from rams at one ($n = 4$; wt), three ($n = 4$; wt), six ($n = 4$; wt), nine ($n = 4$; wt) and 12 ($n = 4$; wt) months of age.

2.1.2. Study design

Testes were collected using local anesthesia in rams 1 to 9 months of age. heart, lung, kidney, *etc*. was collected from 12 mo old rams following exsanguination. The 12-month-old sheep were exsanguinated (in accordance with the requirements of Institutional Animal Care and Use Committee) *via* puncture of the jugular vein, and the heart, liver, spleen, lung, kidney, testis, and epididymal head, body, and tail were removed for further analyses (Shi et al., 2010). Samples (0.5 cm × 0.5 cm × 0.2 cm) of testis and caput, corpus, and cauda epididymis were excised from the 12-month-old sheep, and fixed these with 4% paraformaldehyde. The remaining part of the organs were wrapped in foil, placed in cryotubes, and stored in liquid nitrogen for 3 months, and were subsequently stored at −80 °C. Thickness of sections were 4 μm.

2.2. Total RNA extraction and cDNA synthesis

Tissue samples were removed from −80 °C storage and total RNA was extracted using Trizol reagen (TaKaRa, Dalian, China), according to the manufacturer's instructions. The purity and concentration of total RNA were measured using a Nano-Drop ND-1000 Nucleic Acid/Protein Analyzer (Nanodrop Technologies, Wilmington, DE, USA), and the concentration of RNA samples were adjusted to approximately 1 μg/L, and stored at −80 °C. Reverse transcription was conducted using a PrimeScript® RT Reagent kit (TaKaRa, Dalian, China) by following the manufacturer's instructions. The 10-μL reaction mixtures contained 2 μL of 5 × Prime Script® Buffer (for Real Time; TaKaRa Bio Co. Ltd., Dalian, China), 1 μL of total RNA, and 7 μL of RNase-free ddH_2O. This mixture was allowed to equilibrate at 37 °C for 15 min, and then at 85 °C for 5 s. The resultant products were stored at −20 °C.

2.3. Primer design and synthesis

Primers design was based on human GPR30 mRNA (GenBank accession number CR541741.1) and sheep 18S rRNA (accession number: AY753190.1) sequences, which were used to design a pair of specific primers for each corresponding gene. These primers were synthesized by Beijing BGI Life Tech Co., Ltd. (Beijing, China; sequences are shown in Table 1).

2.4. RT-PCR

The PCR was performed using 15-μL reaction mixtures containing 0.6 μL of each forward and reverse primer (10 μmol/L), 0.8 μL of cDNA template, 5.5 μL of ddH_2O, 7.5 μL of 2 × Taq Master Mix (dye included) (Taq DNA Polymerase, 3 mM $MgCl_2$, 400 μM each dNTP. cat no. CW0682S; KangWei Century Biology Co., Ltd., Beijing, China). Reaction conditions were set as: 35 cycles of 94 °C for 3 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min. The PCR products were detected using electrophoresis with a 3% agarose gel.

2.5. Sequence determination

The recovered DNA fragments were ligated into a T3-cloning-vector according to the instructions provided by manufacturer. The ligation reaction was conducted in a PCR machine, set at 25 °C for 10 min, ensuring that the recovered product could be effectively ligated into the T3-vector (TransGen Biotech., Beijing, China). The ligation product was introduced into DH5α competent cells, which were spread on an ampicillin (AMP)/Luria-Bertani (LB) plate and cultured at 37 °C overnight (12–16 h). Thereafter, white colonies were recovered after a blue/white screening, and cultured in LB liquid medium (containing AMP) for 12–16 h. The cultured bacteria were sent for sequencing at Beijing BGI Life Tech Co. Ltd. (Beijing, China).

2.6. Standard curve for relative quantization

To prepare the standards, a template containing a GPR30 cDNA fragment was diluted eight times, in a series of 2 × dilutions (1.0×2^9; 1.0×2^8; 1.0×2^7; 1.0×2^6; 1.0×2^5; 1.0×2^4; 1.0×2^3; and 1.0×2^2). As a negative control, pure ddH_2O was used. All PCR reactions were performed in duplicate in an Mx3005P real-time thermal cycler (Stratagene, La Jolla, CA, USA) using a SYBR Premix Ex Taq kit (TaKaRa Bio Co. Ltd., Dalian, China) according to manufacturer's instructions. The reaction program was set to 40 cycles of 95 °C for 10 s for denaturation, 95 °C for 5 s, and 60 °C for 25 s. The thermal cycler software generated an