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Characterization and expression of soluble guanylate cyclase in skins and melanocytes of sheep

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ABSTRACT

The study reported the characterization of soluble guanylate cyclase (sGC) with the size of CDS of 1860 bp, encoding a protein of 620 amino acids and containing several conserved functional domains including HNOB, HNOBA, and CHD. Quantitative real time PCR analysis of sGC showed that the expression of sGC mRNA is higher (~5 fold) in white sheep skin relative to black sheep skin with significant difference ($P<0.01$). Using a rabbit polyclonal anti-sGC antibody, an immune reactive band corresponding to sheep sGC protein was detected in the skin samples by Western blotting analysis, and the expression of sGC protein was significantly higher in white sheep skin compared to black sheep skin ($P<0.01$). Immunohistochemical analysis revealed that sGC protein was localized in cytoplasm and intercellular substance of upper hair papilla in hair follicles of white sheep skin, but the protein was localized in cytoplasm and intercellular substance of lower hair bulb and outer root sheath cells in hair follicles of black sheep skin. The immunocytochemical analysis revealed that sGC was expressed in melanocytes *in vitro* of sheep skin. Over expression of sGC in melanocytes resulted in decreased expression of key melanogenic genes including microphthalmia transcription factor (MITF), tyrosinase (TYR), tyrosinase related protein 1(TYRTP1), and tyrosinase related protein 2(TYRP2) both at mRNA and protein level. Moreover, the melanocytes was capable of producing cGMP and cAMP. The observed differential expression and localization of sGC in sheep skins and melanocytes and the capability of producing cGMP and cAMP, which suggested a potential role for this gene in hair color regulation.

1. Introduction

Sheep is one of the most important fiber-producing animals, and the diameter, length and color of fiber are the key traits contributing to the economic value. Currently, different colors of fibers in commercial production are produced by dying white fiber, which affects human health and environment. Thus, natural coat colors in sheep or other fiber-producing species such as alpacas are becoming of increasing interest due to the green revolution and consumer preference for natural products.

Coat colors are determined by both genetics and environment, especially genetics. The phenotype of coat color of animals depends on two types of melanin, black to brown eumelanin and yellow to reddish brown pheomelanin produced in mammalian melanocyte (Ito et al., 2000; Ito and Wakamatsu 2008). The genetic basis for coat color is well understood in rodents (Slominski et al., 2004; Steingrímsson et al., 2006). Some common genes implicated in the regulation of coat color are also well documented in other species including sheep. For example, MC1R and ASIP loci are functionally linked to undesirable coat color phenotypes in sheep (Våge et al., 1999; Norris and Whan 2008), and TYRP1 is a strong positional candidate gene for color variation in Soay sheep (Gratten et al., 2007). In a previous study, we characterized the transcriptome profiles of sheep skins with white and black coat color, and identified differentially expressed genes (Fan et al., 2013) including known coat color genes (*e.g.*, DCT, MATP, TYR and TYRP1). One of the differentially expressed genes is soluble guanylate cyclase (sGC), which showed significantly higher expression in white *vs*. black sheep skin.

Guanylate cyclases are a family of enzymes that catalyze the conversion of GTP to cGMP. The family comprises both membrane-bound (particulate guanylate cyclase, pGC) and soluble (soluble

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guanylate cyclase, sGC) isoforms that are expressed in nearly all cell types (Fan et al., 2013). sGC is involved in many signal transduction pathways, most notably in the cardiovascular system and the nervous system (Denninger and Marletta 1999). The present study reports the characterization of sGC expression in sheep skins with white and black coat color, providing evidence to suggest that sGC might be related to coat color formation.

2. Materials and methods

2.1. Sheep and tissue collection

Housing and care of sheep and collection of skin samples for use in the described experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms.ch/index.php/12-newsflash/365-announcement) and approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University, Taigu, China. Six healthy 2-year-old male sheep with white and black coat color phenotype (3 sheep per color) were used in the study, which were from the sheep farm in Sunite, Inner Mongolia, China. Skin samples of 8 mm diameter from the back of sheep were collected. For each sample, part of the tissue was flash frozen in liquid nitrogen and stored at −80 °C until RNA and protein extraction, and the remaining part was fixed in 4% formaldehyde for paraffin sections.

2.2. Construction of plasmids

The coding sequence of sheep sGC was obtained by PCR using sheep skin cDNA as a template with primers containing ScaI and XhoI sites (Table 1). The PCR product of sGC and the pmirGLO vector (Promega, Fitchburg, WI) were digested with ScaI and XhoI and then ligated together to obtain the pmirGLO-sGC construction.

2.3. Cell culture and transfection

All melanocyte cell cultures of sheep used in this study were established by our lab. Skin samples used for established the melanocyte lines were obtained from the sheep which were from the sheep farm in Sunite,Inner Mongolia, China. Cell were transfected with the pmirGLO-sGC plasmid using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three days after transfection, melanocytes were collected. Total RNA and Cell lysates were prepared and subjected to real-time PCR and Western blot analyses, respectively.

2.4. Quantitative real time PCR analysis

Total RNA was isolated from sheep skin samples using Trizol reagent (Invitrogen). The concentration of total RNA was determined using NanoDrop 1000 spectrophotometer (NanoDrop Technology). Prior to reverse transcription, total RNA was treated with DNaseI (Ambion) to eliminate genomic DNA contamination. Two microgram of treated total RNA from each sample were converted to cDNA using Prime Script™ RT reagent kit (TaKaRa). Negative control RT reactions were also performed at the same time. Real-time PCR primers for sGC, MITF, TYR, TYRP1, and TYRP2 gene and the endogenous control gene (β-actin) were designed using primer3plus software (http://www.biowebdb.org/primer3plus/, see Table 1). Quantitative real time PCR was performed using SYRB Premix Ex Taq™ II (TaKaRa) in triplicate for each sample on a 7500 Fast Real-Time PCR System (Applied Biosystems). Quantification of sGC, MITF, TYR, TYRP1 and TYRP2 transcript abundance was performed using the comparative threshold cycle (CT) method (Livak and Schmittgen 2001). The relative amount of mRNA was normalized to the amount of β-actin mRNA.

Table 1
Primers used in this study.

Primer name	Primer sequence 5′-3′	Application
sGC-F1	GGTCAAGGAAGAGGTCGACG	Real time PCR
sGC-R1	GATGACTGGTGTCTCCCGGT	Real time PCR
sGC-F2	CGAGCTCCAGATGATCTACTTGCCTGAA	RT-PCR
sGC-R2	GCTCTAGATATCTGAACAGATTCACCGTCTACT	RT-PCR
β-actin-F	CCTGACCGGGAAGAGGAATC	Real time PCR
β-actin-R	AAAAACAGGGGGTTGAACTC	Real time PCR

2.5. Immunohistochemistry

Paraffin sections were dehydrated with increasing concentrations of ethanol (80–100%), followed by incubation in 3% hydrogen peroxide for 10 min at room temperature to block the action of any endogenous peroxidase. After washing with 0.1 M PBS three times for 15 min, the sections were boiled for 10 min in 0.01 M citric acid. This was followed by a 20-min immersion in PBS containing 5% BSA at 37 °C. Sections were then incubated at 4 °C overnight in 1:100 diluted rabbit anti-sGC primary antibody (Abcam, ab53084, USA, used for WB, IHC-P). Following three times of washing in PBS, sections were incubated in 1:500 diluted HRP conjugated goat anti-rabbit IgG (CWBIO, Beijing, China) for 30 min at 37 °C. After washing with PBS three times, sections were developed with DAB and the positive signal was observed using a Leica microscope (Leica Microsystems). PBS was substituted for the primary antibody for the negative control.

2.6. Western blotting

Total protein extraction from sheep skins was performed using WB/IP lysis buffer following the manufacturer's instruction (Beyotime, Beijing, China). Protein concentrations were measured by the BCA method using bovine serum albumin as the standard. Protein extracts were denatured at 95 °C for 5 min, and the same amount of protein (150 μg) from each sample was separated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunoblotting was carried out with 1:200 diluted rabbit anti-sGC antibody(Abcam, ab53084, USA, used for WB, IHC-P), 1:500 diluted rabbit anti-MITF antibody (Abcam, ab20663, USA, used for ICC/IF, WB), 1:500 diluted rabbit anti-TYR antibody (Abcam, ab180753, USA, used for ICC/IF, WB, IHC-P), 1:500 diluted rabbit anti-TYRP1 antibody (Abcam, ab73873, USA, used for WB, IHC-P), 1:500 diluted rabbit anti-TYRP2 antibody (Abcam, ab74073, used for IHC-P, WB, ELISA) and 1:1000 diluted rabbit anti-β-actin antibody (CWBIO, CW0097, Beijing, China,used for WB, ELISA) in 5% milk blocking buffer at 4 °C overnight. After washing in TBST, the membrane was incubated with HRP conjugated goat anti-rabbit IgG (CWBIO, Beijing, China) for 1 h at room temperature (1:2000 diluted in 3% blocking buffer). Following several washings with TBST, the signals were detected using an ECL kit (CWBIO, Beijing, China). The intensity of the signals for sGC and β-actin was qualified using Image-Pro Plus Software (Olympus).

2.7. Immunocytochemistry

In order to investigate the sGC expression in melanocytes, melanocytes were washed three times in 0.1 M PBS for 3 min each, fixed in 4% paraformaldehyde, and then incubated at room temperature in 3% hydrogen peroxide for 15 min to block the action of any endogenous peroxidase. After washing with 0.1 M PBS three times for 5 min each, cells were immersed in BSA at 37 °C for 40 min. Cells were then incubated at 4 °C overnight in anti-sGC antibody(Abcam, ab53084, USA, used for WB, IHC-P) solution. Following washing