Expression of the \textit{GPRC5D} gene in Liaoning Cashmere goats

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\textbf{1. Introduction}

Liaoning Cashmere goats are a precious genetic resource of China, and their export is banned. Cashmere production has great economic value and China is the largest producer of cashmere. In particular, cashmere yield and quality greatly influence the economic benefit derived from Cashmere goat breeding. Therefore, it is beneficial to investigate the biological characteristics of any genes that may be involved in regulating cashmere growth. Cashmere goat heterogeneous fleece mainly comprises unmedullated cashmere and medullated wool. The skin hair follicles are divided into 2 categories, the first growing the wool, or the primary hair follicles, and the second growing cashmere, or the secondary hair follicles. After the wool follicles become mature and active, they go through 3 regular phases, the anagen, catagen, and telogen phases, during a year’s cycle. This triphasic activity is mainly exhibited by the secondary follicles. Although the primary follicles have periodic variation over the year, the cycle is not obvious. Even during the telogen phase, hair follicles are still growing. Therefore, controlling the length of the anagen period could control the cashmere yield.

In a previous study, cDNA library construction was executed using total RNA isolated from skin during follicle anagen and a full-length cDNA clone was isolated for \textit{G-protein-coupled receptor, family C, group 5, member D (GPRC5D)}. Lefkowitz and Kobilka, who won the Nobel Prize in Chemistry of 2012, indicated that GPRCs are the detectors from which the sensory receptor cells directly receive external signals (1). \textit{GPRC5D} is a member of the retinoic acid inducible gene-1 (RAIG-1) family, which comprises 4 genes (\textit{RAIG1}, \textit{GPRC5B}, \textit{GPRC5C}, and \textit{GPRC5D}) (2). The RAIG-1 gene family is thought to influence cell growth and differentiation (3). Retinoic acid (RA) affects the growth of hair follicles and several RA receptors are expressed in hair follicles. Analyses using a retinoid receptor antagonist showed that deficient receptor function could inhibit hair growth (4,5). Bazzano et al’s analyses of mouse and human hair follicles suggested that RA prolonged the anagen phase and shortened the telogen phase (6). Shinichi et al. showed that \textit{GPRC5D} is present in mammalian skin and is related to epidermis keratinization (3). However, there are no research reports concerning this gene in Cashmere goats. Therefore, to further study the biological characteristics of this gene, we first analyzed the bioinformatics of the \textit{GPRC5D} gene. In addition, RT-PCR was used to analyze the expression of the \textit{GPRC5D} gene. Lastly, the specific expression sites of the \textit{GPRC5D} gene were detected by in situ hybridization. These findings lay a solid foundation for further study of the regulatory mechanism of the \textit{GPRC5D} gene.
2. Materials and methods

2.1. Sample collection
The samples were obtained from a new-breeding cashmere goat farm in Wafangdian, Liaoning. We chose 4 adult goats (2 bucks and 2 nannies) of 1.5 years old as the experimental sample. In the last 10 days of October, the goats were slaughtered and the skin, heart, liver, spleen, lung, and kidney tissues were collected and placed in liquid nitrogen for preservation.

2.2. GPRC5D gene sequence analysis and bioinformatics
The sequence of GPRC5D (GenBank accession no. KC981077) was analyzed by BLAST to identify amino acid and nucleic acid homology, and the open reading frame (ORF) was identified using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A phylogenetic tree was constructed using a multiple alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) of GPRC5D gene sequences. The amino acid composition, molecular weight, isoelectric point, and physicochemical properties were analyzed using ProParam. PredictionProtein was used to predict the secondary structure and domain. ProtScale was used to assess the hydrophobicity of GPRC5D and the TMHMM program was used to predict transmembrane domains. All the above analyses and predictions were performed in the Expert Protein Analysis System (http://www.expasy.org/). Signal peptide prediction was performed by the CBS servers (7).

2.3. Semiquantitative RT-PCR detection
Total RNA was extracted from skin, heart, liver, spleen, lungs, and kidneys during the anagen phase using TRIzol Reagent (Invitrogen, USA). RNA was stored at −80 °C. Reverse transcription was performed following the RT-PCR kit instructions (TianGen, Dalian, China). A GPRC5D-specific primer was designed according to the cDNA, 12.5 µL of 2X Master Mix (TaKaRa, Dalian, China), 1 µL of each primer F/R, 2 µL of RT products, and 8.5 µL of deionized water. The reaction conditions were 2min at 94°C, 30s at 55°C, 30 s at 72°C, and then 72°C for 60 min. To detect the PCR products, 1% agarose gel electrophoresis was used. The results were scanned and analyzed using the Gel-Pro Analyzer 4 program.

Table. Reaction primer of PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
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<tr>
<td>GPRC5D</td>
<td>-F 5'-ACGCAGCTTCCAGAGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>-R 5’-CCTTTAGCCCGTGGATGAG-3'</td>
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2.4. In situ hybridization
After cloning and sequencing, RT-PCR products of the GPRC5D gene were purified to generate a concentration of GPRC5D cDNA product that could be labeled with digoxigenin. Labeling was performed with a digoxigenin kit (Roche, Germany) according to the manufacturer's instructions. The prepared probes were stored at −20 °C. Tissue sections for in situ hybridization were obtained via dewaxing, rehydration, proteinase K digestion, and drying. Prehybridization was performed for 1 h with 50% deionized formamide, and then hybridization was performed at 42 °C overnight. The hybridized sections were washed successively in 2X SSC, 1X SSC, and 0.25X SSC before being detected immunologically using antiantibodies from sheep (Roche). Antibody binding was visualized using a color-substrate solution (8-10). The slides were sealed under a neutral resin seal sheet, observed under a microscope BA310 (Motic, China), and photographed.

3. Results

3.1. Bioinformatic analysis of the GPRC5D gene sequence
The GPRC5D cDNA was 1278 bp long and contained an intact ORF of 903 bp encoding a putative protein of 300 amino acids. There were 9 initiator codons.

Moreover, bioinformatic prediction of the protein primary structure indicated that GPRC5D had a high proportion of leucine and valine. Protein secondary structure prediction indicated that GPRC5D had a high proportion of α-helices (60.33%) and that the rest of the structure was extended strand (10%) and random coil (29.67%). The GPRC5D protein comprised a putative conserved 7tm_3 (7-transmembrane domain). No signal peptide was identified in GPRC5D. Phosphorylation site prediction identified 3 Ser, 2 Try, and 1 Thr potential phosphorylation sites in the GPRC5D protein.

3.2. RNA extraction
Agarose gel electrophoresis showed that total RNA extracted from the skin, heart, liver, spleen, lungs, and kidneys of Liaoning Cashmere goat was relatively intact (Figure 4).

3.3. RT-PCR
RT-PCR analysis of samples from skin, heart, liver, spleen, lungs, and kidneys of Liaoning Cashmere goat showed that the GPRC5D gene was only expressed in the skin; no expression was observed in the heart, liver, spleen, lungs, or kidneys (Figure 5).
3.4. In situ hybridization

The in situ hybridization results showed a strong GPRC5D signal in the inner root sheath (IRS) of primary and secondary hair follicles, but not in the hair shaft, outer root sheath (ORS), or follicular papilla (FP). Each experiment had a separate control, in which no expression signal was found (Figure 6).

4. Discussion

The Liaoning Cashmere goat is native to the Liaodong Peninsula and is a high-yield Cashmere goat (11). The Liaoning Cashmere goat has a particularly fine, variable Cashmere fleece (12). Cashmere has a very good textile performance, which is closely related to increased cashmere yield (13). Some scholars have studied GPRC5D of the RAIG-1 family in man and rat (3,14). However, this gene has not been studied in the Cashmere goat. GPRC5D is a 7-transmembrane receptor. After binding with its ligand, GPRC5D acts through a coupled G-protein to increase or decrease secondary messenger activity, which changes the ion channel on the membrane, resulting in changes to the membrane electric potential (15–17). Therefore, adjusting and controlling the GPRC5D gene could effectively regulate the system with which it is associated, so that it can develop in a direction that is beneficial for the industry.

Bioinformatic analysis can predict the structure of a gene and protein and can lay a foundation for further study of the protein's function. GPRC5D of the Liaoning Cashmere goat showed high homology to the same protein from Bos taurus, Canis lupus, Sus scrofa, Equus caballus, Oryctolagus cuniculus, Callithrix jaccus, Loxodonta africana. However, it showed low homology to the protein from Equus caballus. The phylogenetic tree confirmed these results. In addition, prediction of transmembrane domains in GPRC5D using TMHMM (Figure 1) indicated that it has 7 transmembrane domains, suggesting that the protein is a membrane receptor or located on the cell membrane. Primary structure prediction showed that it has a high leucine and valine

Figure 1. Prediction and analysis of transmembrane domains of GPRC5D using TMHMM.

Figure 2. Evolutionary analysis of GPRC5D of Liaoning cashmere goat in comparison with the GPRC5D sequences of Bos taurus, Canis lupus, Sus scrofa, Equus caballus, Oryctolagus cuniculus, Callithrix jaccus, Loxodonta africana.
Figure 3. Multiple sequence alignment of amino acid sequences of GPRCSD from Liaoning cashmere goats with that of Bos Taurus, Canis lupus, Sus scrofa, Equus caballus, Oryctolagus cuniculus, Callithrix jacchus, and Loxodonta africana. Accession numbers of genes are XM_002712647.1, XM_002752172.1, XM_002712647.1, XM_002752172.1, XM_001496882.1, XM_003405342.1, XM_543806.3, XR_135495.1 and NM_001105412.1. Residues that match the Cashmere sequence are shaded. Black boxes denote in-frame domains and α-helices are shown in bold. Asterisks below the alignment indicate sequence identity. Colons denote conservative substitutions, while dots show nonconservative substitutions.
content, which are hydrophobic amino acids that fit closely with the hydrophobicity and transmembrane analysis.

RT-PCR analysis showed that \textit{GPRC5D} was only expressed in the skin of Cashmere goats. Inoue et al. used RT-PCR analysis on human samples and found that \textit{GPRC5D} was only present in skin (3). Thus, we can conclude that \textit{GPRC5D} is specifically expressed in the skin. \textit{GPRC5D} could influence the growth and develop of the skin cells of Cashmere goat (18, 19) and thus influence the quality of cashmere.

In situ hybridization results showed a strong signal for \textit{GPRC5D} in the IRS of primary and secondary hair follicles, but not in the hair shaft (Co, Cu, and Med), ORS, or FP. The location of \textit{GPRC5D} in the IRS agrees with its suggested association with cashmere drop-off. Inoue et al. showed that \textit{GPRC5D} had a close relationship with cashmere growth and could prolong the anagen phase of hair follicles (3,20). Prolonging the anagen phase of hair follicle growth by adjusting this gene and reducing the telogen could provide the theoretical foundation for increasing cashmere yield.

In summary, we analyzed the protein features and gene expression of \textit{GPRC5D} by bioinformatics. We predicted its protein structure and function and examined its expression in different tissues and phases of follicle growth. The results presented here lay a solid foundation for further study of the function of the \textit{GPRC5D} gene in regulating hair follicle growth and hint at the molecular basis of the functions of genes involved in regulating the diameter of fluffy fibers.

\textbf{Acknowledgments}

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Figure 6. The expression of the GPRC5D gene in the Liaoning cashmere goat hair follicles. Panels A, B, and C show the control group (100×); panels D, E, and F show GPRC5D expression in transverse and longitudinally cut hair follicles (100×). IRS, inner root sheath; ORS, outer root sheath; Med, medulla; CO, cortical layer; Cu, cuticle; FP hair follicle papilla.
References


