TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2014) 38: 347-353
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doi:10.3906/vet-1310-35

Expression of the *GPRC5D* gene in Liaoning Cashmere goats

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Received: 14.10.2013 • **Accepted:** 21.04.2014 • **Published Online:** 17.06.2014 • **Printed:** 16.07.2014

Abstract: This study aimed to characterize the expression the *GPRC5D* gene in the skin of the Liaoning Cashmere goat. Initially, the *GPRC5D* gene sequence was subjected to bioinformatics analysis. Second, semiquantitative RT-PCR was used to detect *GPRC5D* gene expression in the tissues. Third, in situ hybridization was used to locate *GPRC5D* gene expression in the primary and secondary hair follicles. Functional site analysis showed that *GPRC5D* has 7 transmembrane domains. RT-PCR showed that *GPRC5D* was expressed only in the skin. In situ hybridization showed that *GPRC5D* was expressed in the inner root sheath only. We speculated that *GPRC5D* may regulate cashmere growth.

Key words: *GPRC5D*, bioinformatics, RT-PCR, in situ hybridization

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 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). *GPRC5D* is a member of the retinoic acid inducible gene-1 (RAIG-1) family, which comprises 4 genes (*RAIG1*, *GPRC5B*, *GPRC5C*, and *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 *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 *GPRC5D* gene. In addition, RT-PCR was used to analyze the expression of the *GPRC5D* gene. Lastly, the specific expression sites of the *GPRC5D* gene were detected by in situ hybridization. These findings lay a solid foundation for further study of the regulatory mechanism of the *GPRC5D* gene.

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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. The NetPhos program predicted phosphorylation sites. 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 sequence shown in the Table. The cDNA products were stored at –20 °C.

The PCR reaction system (25 μL) was as follows: 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 94 °C for 3 min and then 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s for 30 cycles, followed by 72 °C for 5 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.

Gene name		Sequence
GPRC5D	-F	5'- ACGCAGCTTCAGAGCAGAGA-3'
	-R	5'-CCTTTAGCCCGTGGAATGAG -3'

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.

Hydrophobicity and transmembrane domains of *GPRC5D* are shown in Figure 1. Evolutionary analysis of *GPRC5D* is shown in Figure 2. Multiple sequence alignment of amino acid sequences of *GPRC5D* are shown in Figure 3.

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).

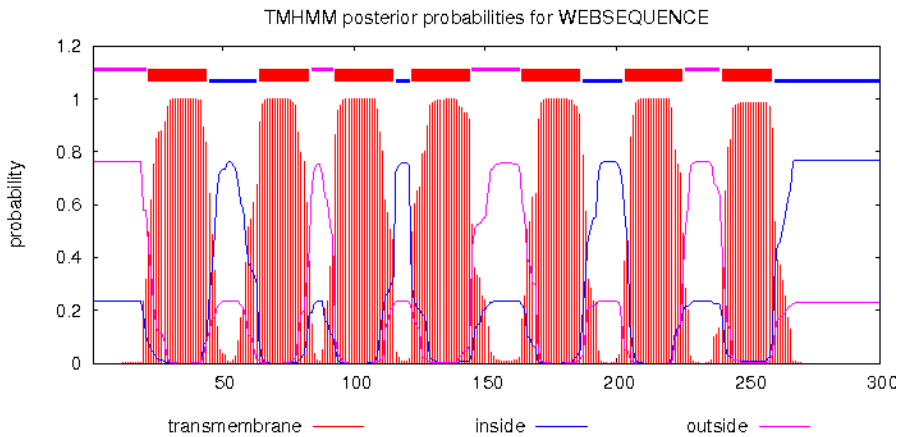

Figure 1. Prediction and analysis of transmembrane domains of *GPRC5D* using TMHMM.

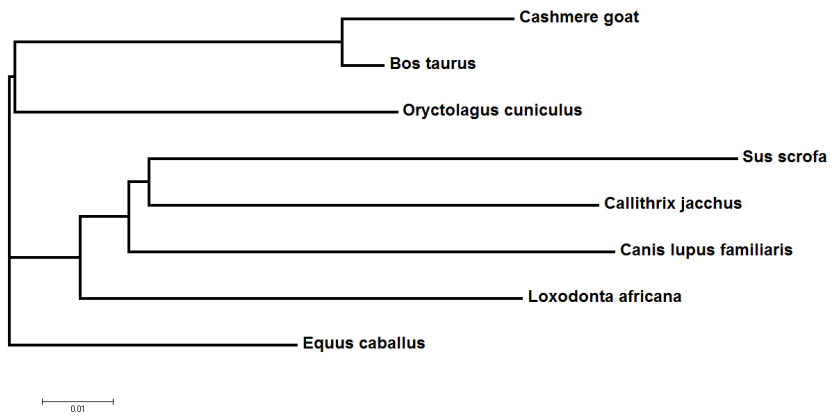

Figure 2. Evolutionary analysis of *GPRC5D* of Liaoning cashmere goat in comparison with the *GPRC5D* sequences of *Bos taurus*, *Canis lupus*, *Sus scrofa*, *Equus caballus*, *Oryctolagus cuniculus*, *Callithrix jacchus*, *Loxodonta africana*.

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*, and, to a lesser degree, to that of *Oryctolagus cuniculus*. 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

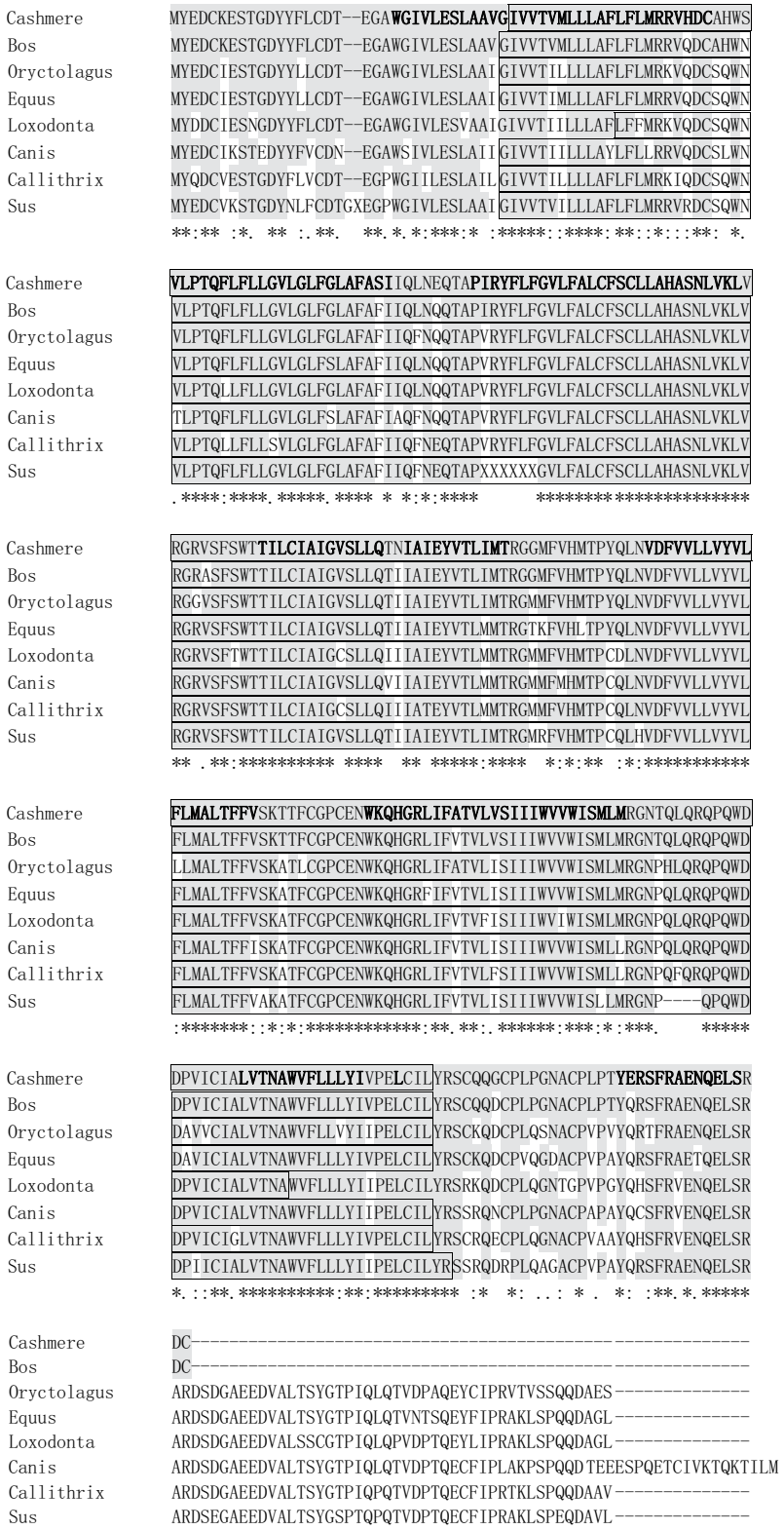

Figure 3. Multiple sequence alignment of amino acid sequences of *GPRC5D* from Liaoning cashmere goats with that of *Bos Taurus*, *Canis lupus*, *Sus scrofa*, *Equus caballus*, *Oryctolagus cuniculus*, *Callithrix jacchus*, and *Loxodonta africana*. Accession numbers of genes are XM_002712647.1, XM_002752172.1, XM_001496882.1, XM_003405342.1, XM_543806.3, XR_135495.1 and NM_001105412.1. Residues that match the Cashmere *GPRC5D* 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.

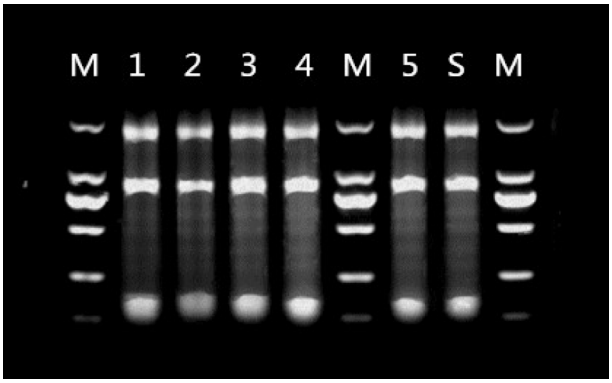

Figure 4. RNA of *GPRC5D* gene analysis from heart, liver, spleen, lungs, kidneys, and skin. M, DL500 ladder; 1, heart; 2, liver; 3, spleen; 4, lung; 5, kidney; S, skin.

content, which are hydrophobic amino acids that fit closely with the hydrophobicity and transmembrane analysis.

RT-PCR analysis showed that *GPRC5D* was only expressed in the skin of Cashmere goats. Inoue et al. used RT-PCR analysis on human samples and found that *GPRC5D* was only present in skin (3). Thus, we can conclude that *GPRC5D* is specifically expressed in the skin. *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 *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 *GPRC5D* in the IRS agrees with its suggested association with cashmere drop-off. Inoue et

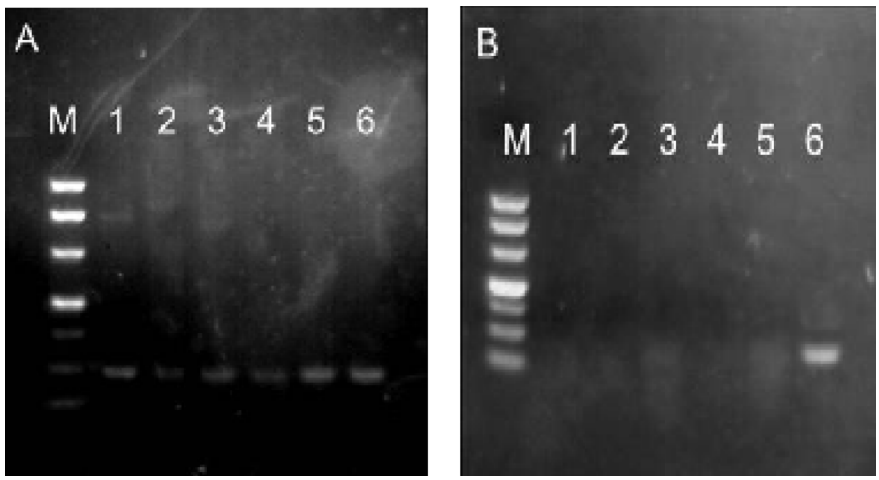

Figure 5. Crystal structures of β-actin and RT-PCR *GPRC5D* gene analysis in heart, liver, spleen, lungs, kidneys, and skin. A: β-Actin; B: *GPRC5D* gene. M, DL500 ladder; 1, heart; 2, liver; 3, spleen; 4, lung; 5, kidney; 6, skin.

al. showed that *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 *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 *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.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (No. 31172188), Technical Plan Project of Dalian (No. 2013B12NC090), Distinguished Professor Subsidy Scheme in Liaoning Province and Science and Technology Innovation Project of the Chinese Academy of Agricultural Sciences (CXGC-IAS-13-2014),and the Agricultural Science and Technology Innovation Program of China (No. ASTIP-IAS13).

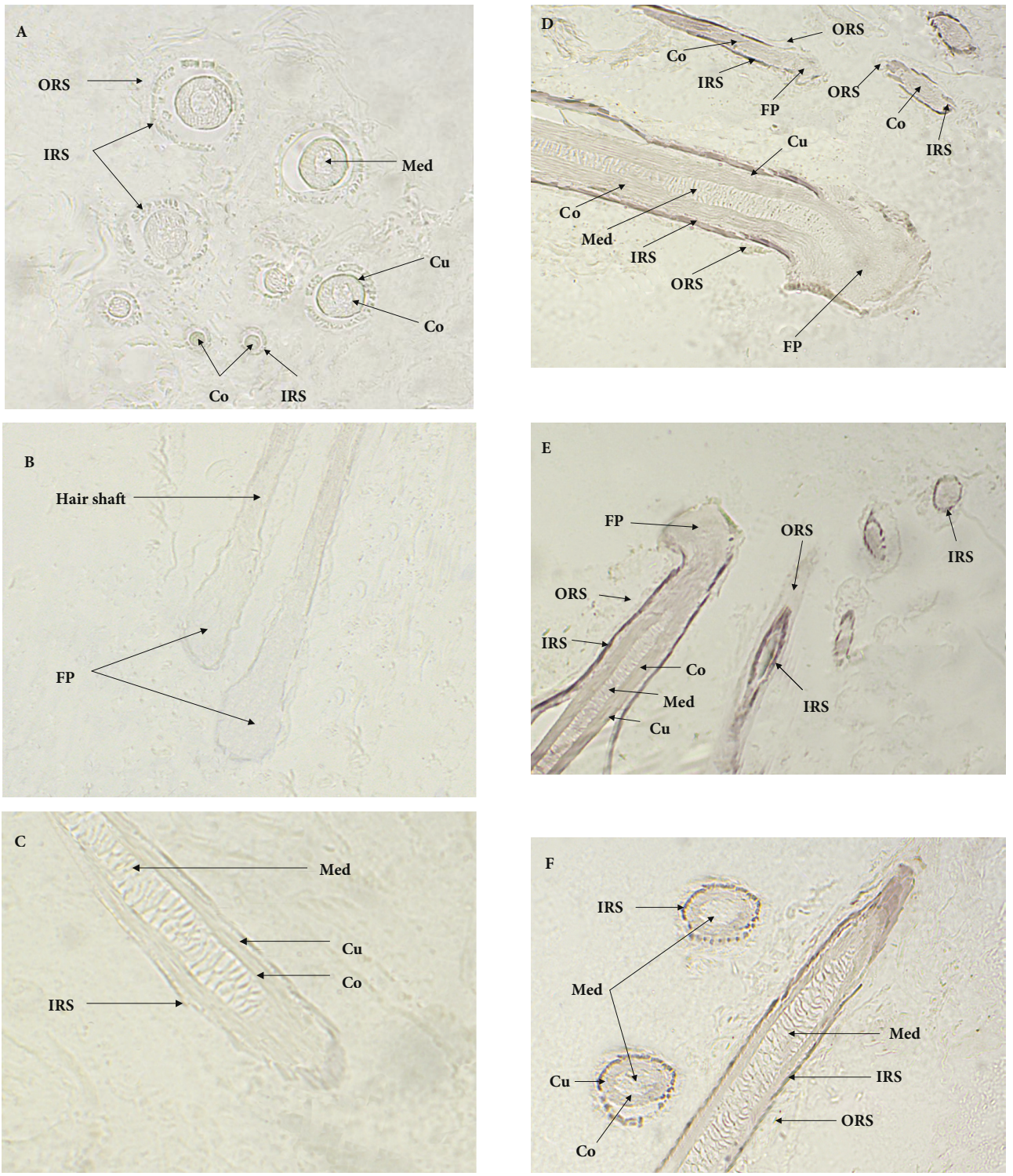

Figure 6. The expression of the *GPRC5D* gene in the Liaoning cashmere goat hair follicles. Panels A, B, and C show the control group (100×); panels D, E, and F show *GPRC5D* expression in transverse and longitudinally cut hair follicles (100×). IRS, inner root sheath; ORS, outer root sheath; Med, medulla;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

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