Regulatory role of LEF-1 in the Proliferation of Arbas White Cashmere Goat Dermal Papilla Cells

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Regulatory role of *LEF-1* in the Proliferation of Arbas White Cashmere Goat Dermal Papilla Cells

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**Abstract**

Cashmere, which has high economic value, is made from the secondary hair follicles of cashmere goat skin. Dermal papilla cells (DPCs) are considered the center of regulation of hair growth, which is closely related to hair follicle growth. We constructed *LEF-1* overexpression and interference experiment groups of goat DPCs to investigate *LEF-1* regulation of DPCs proliferation by Wnt signaling, and provide a theoretical basis for improving cashmere yield. In primary DPCs, *LEF-1*, *β-catenin*, *C-myc*, and *cyclin D1* expression in the *LEF-1* overexpression group was 9.25-, 1.27-, 1.74-, and 1.63-fold, respectively, that of the control. *LEF-1*, *β-catenin*, *C-myc*, and *cyclin D1* expression in the *LEF-1* interference group was 0.20-, 0.75-, 0.38-, and 0.39-fold, respectively, that of the control. In secondary DPCs, *LEF-1*, *β-catenin*, *C-myc*, and *cyclin D1* expression in the *LEF-1* overexpression group was 10.53-, 1.48-, 1.64-, and 1.39-fold, respectively, that of the control. *LEF-1*, *β-catenin*, *C-myc*, and *cyclin D1*
expression in the LEF-1 interference group was 0.21-, 0.71-, 0.40-, and 0.36-fold, respectively, that of the control. Primary and secondary DPCs proliferation rates changed with LEF-1 expression. Therefore, the LEF-1 regulation pattern of cell proliferation through Wnt signaling is similar in both DPCs.

Keywords: Cashmere goat, Dermal papilla cell, LEF-1.

INTRODUCTION

The Cashmere goat skin hair follicle is classified into primary hair follicles and secondary hair follicles based on their time of occurrence and structural characteristics. Primary hair follicles develop to form wool while the secondary hair follicles develop to form cashmere. (Ji et al. 2016; Qiao et al. 2016; Shi et al. 2016). The hair follicle is a complex skin subsidiary organ that controls hair growth (Powell et al. 1992). The hair follicle can be divided into two main parts, the portions that are part of the epithelium and the dermis, and is composed of five segments: the hair ball, hair shaft, inner root sheath, outer root sheath, and connective tissue sheath (Ji et al. 2016; Xing et al. 2011). A hair ball is composed of the dermal papilla and hair matrix. The dermal papilla cells (DPCs) are located at the base of the hair follicle, and they secrete a variety of cytokines to regulate the adjacent tissues, thereby regulating the growth and renewal of hair (Jeong et al. 2015). Accordingly, the DPCs have been referred to as the “mesenchymal command center” of the hair follicle (Morgan 2014). Thus, the primary and secondary DPCs from cashmere goats may represent valuable cell models for studying hair follicle growth and cyclical changes, including investigations of the regulatory mechanisms of the various genes involved in hair follicle growth and development.
The Wnt signaling pathway regulates cell proliferation, differentiation, and apoptosis through signal transduction between cells and plays a key regulatory role in mammalian embryonic development and organ formation. The Wnt signaling pathway includes both classical and non-classical signaling pathways. The classical Wnt signaling pathway triggers the transcription of β-catenin/TCF enhancers and upregulates the expression of target genes through accumulation of β-catenin in the cytoplasm. The non-classical signaling pathway does not depend on β-catenin, which is involved in the cellular polarity signaling pathway and Wnt/Ca\(^{2+}\) pathway. However, the proliferation and differentiation of hair follicles are mainly mediated through the classical Wnt signaling pathway (Xiong et al. 2014). In mice, relevant studies on the growth and development of hair follicles have been reported. For example, conditional knockout of β-catenin in embryonic skin blocked the development of hair follicles resulting in no new hair formation (Osada and Kobayashi 2000). In addition, specific knockout of the β-catenin nuclear conjugate LEF-1 resulted in follicle development termination (Andl et al. 2002). However, in transgenic mice stably expressing β-catenin, a large number of nascent hair follicles appeared in the epithelium, which eventually led to the emergence of hair follicle tumors (Gat et al. 1998; Lo Celso et al. 2004). Furthermore, LEF-1 overexpression could promote the growth of hair follicles (Amberg et al. 2016; Leiros et al. 2017). Moreover, regeneration of new hair follicles at a wound site was found to depend on the activation of the Wnt signaling pathway. However, to our knowledge, the roles of LEF-1 and β-catenin have not yet been addressed in the DPCs of cashmere goats. Therefore, it is necessary to clarify the mechanisms underlying the growth and
periodic regulation of the hair follicle, which could provide breeding and genetic strategies to improve wool and cashmere production.

In the present study, the primary and secondary DPCs of an Arbas White Cashmere goat were used as cell models to provide experimental evidence for exploring the mechanism of signal regulation related to the growth of the hair follicle.

MATERIALS AND METHODS

Culture of dermal papilla cells

The samples were collected from a cashmere goat of an Arbas White Cashmere goats breeding farm in the Inner Mongolia Autonomous Region of China. Skin samples (1–2 cm²) were collected in September, which is the season of hair follicle growth, from a 2-year-old female Arbas White Cashmere goat. The primary and secondary dermal papilla cells were isolated as described previously (Zhu et al. 2013). The separated cells were cultured in Dulbecco’s modified Eagle medium/F12 containing 10% fetal bovine serum in a 5% CO₂, 37°C incubator.

Immunocytochemistry

Immunocytochemistry was carried out to confirm the identity of the isolated cells. The second-generation primary and secondary DPCs were cultured on coverslips placed in culture dishes. The cells were washed three times with 0.01 M phosphate-buffered saline (PBS) and grown to 90% confluence. The cells were then incubated with 4% paraformaldehyde at 4°C for 30 min, and the immobilized cells were treated with 0.5%
Triton X-100 for 10 min at room temperature, washed three times with 0.01 M PBS, and incubated with PBS containing 5% bovine serum albumin at room temperature for 60 min to minimize non-specific staining. The cells were incubated overnight at 4°C with the primary antibody against alpha-smooth muscle actin (α-SMA; 1:100 diluted; Boster Biotech) and CD133 (1:100 diluted; Boster Biotech), washed three times with 0.01 M PBS, and then incubated again for 60 min at 37°C with the secondary antibody, goat anti-rabbit/mouse IgG (1:100 diluted; Boster Biotech). Finally, the cells were stained with 1 mg/L 4',6'-diamidino-2-phenylindole (DAPI; Boster Biotech) solution for 5 min after washing again with 0.01 M PBS. The fluorescence images were obtained after inversion of the fluorescence microscope.

**Plasmid construction and transient transfections**

The amplified *LEF-1* gene coding sequence and cytomegalovirus promoter were added to the pDsRed2-1 skeleton plasmid through an experimental molecular biology technique to achieve the overexpression vector named pDsRed2-LEF-1. The RNA interference vector of *LEF-1* was constructed with pSIREN-RetroQ-ZsGreen as the backbone. The interfering RNA sequence of the *LEF-1* gene design was 5’-GGACCCTCTTATTGAGTTT-3’. The constructed vector was confirmed by sequencing, and the primary and secondary DPCs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested for subsequent real-time polymerase chain reaction (PCR) and western blotting analyses.
Growth curve of dermal papilla cells

The cells at a good growth state were digested with trypsin, and then the cell number in the suspension was counted in new medium using a hemocytometer. Based on the cell count results, the cells were subcultured at a density of $5 \times 10^4$ cells/mL. After 24 h of incubation, cell counting was initiated to construct the growth curve. The counting process was performed every 24 h for 7 consecutive days. Three groups of the same cultured cells were counted at each time point, and the mean value was calculated and used to plot the growth curve, with cell number on the vertical axis and time on the horizontal axis.

EdU incorporation assay

The proliferation of DPCs was examined via the 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay using the EdU Assay Kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. DPCs were seeded in 24-well cell culture plates at $2 \times 10^4$ cells per well and cultured for 48 h at 37 °C. The cells were further incubated with 50 µM EdU medium for 3 h at 37°C. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature and treated with 0.5% Triton X-100 for 10 min at room temperature. After washing the cells three times with PBS, 1× Apollo® staining reaction solution was added, and the cells were incubated at room temperature for 30 min. Finally, the cells were treated with Hoechst 33342 for 30 min. The fluorescence images were obtained after inversion of the fluorescence microscope. EdU-positive cells (red cells) and Hoechst 33342-positive cells (blue cells) were counted by using Image J.
software (National Institutes of Health, USA), and the EdU incorporation rate was calculated. The EdU incorporation rate is the ratio of the number of EdU-positive cells to the number of Hoechst 33342-positive cells. All experiments were performed in triplicate, with three independent replicates.

**Real-time PCR**

Total RNA was extracted from the lysates of the cells in each experimental group (LEF-1-overexpressing, LEF-1-interference, and control) using RNAiso Plus reagent (TaKaRa Biotechnology, Dalian, China). The genomic DNA was then removed by treatment with gDNA Eraser to reduce its influence on the experimental results. Equal amounts of each RNA sample were used to synthesize cDNA by using the PrimeScript RT reagent Kit (TaKaRa Biotechnology) according to the manufacturer’s instructions. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal reference gene. The primers for amplifying LEF-1, β-catenin, C-myc, cyclin D1, and GAPDH were designed and are shown in Table 1.

Real-time PCR was performed using the iQ5 system (BioRad, Hercules, CA, USA) with SYBR Premix Ex Taq (TaKaRa) as directed by the manufacturer. The real-time PCR cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. The specificity of each primer was determined by the melting curve of the real-time PCR. The results were obtained after three independent experiments with three replicates each. The relative quantitative analysis of each gene was performed by using the comparative cycle threshold (Ct) value method. The relative expression level
of each gene was calculated by using the $2^{-\Delta\Delta\text{Ct}}$ method.

**Western blot analysis**

The proteins of the respective cell lines were extracted. Equal amounts of each protein were transferred to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein membrane, and the membrane was incubated at 4°C with the primary antibody against LEF-1 (1:500 dilution; Abcam), C-myc (1:500 dilution; Abcam), cyclin D1 (1:500 dilution; Abcam), and β-catenin (1:500 dilution; Abcam); horseradish peroxidase-tagged IgG antibody was used as the secondary antibody. A β-actin antibody at 1:1,000 dilution was used as a loading control. The protein bands were visualized using the Tanon-5200 image analysis system.

**Statistical analysis**

The data were obtained from three independent experiments, and each experiment was performed with three replicates. The experimental data are presented in the form of mean ± standard deviation, and were analyzed using Graphpad Prism 6 software. Data between groups were compared with the $t$-test, and a $P$-value less than 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

*In vitro culture and identification of Arbas White Cashmere goat dermal papilla cells*
Upon in vitro culture, the primary and secondary DPCs of an Arbas White Cashmere goat showed a spindle-shaped morphology, with adherent growth (Fig. 1A). The cells showed agglutination growth in vitro for 5–7 days. The cells were positive for α-SMA and CD133 markers (Fig. 2), confirming that they were indeed DPCs.

**Plasmid transfection into the primary and secondary dermal papilla cells**

The *LEF-1*-overexpression and -interference vectors were respectively transfected into both the Arbas White Cashmere goat primary and secondary DPCs by using liposomes, and the transfection efficiency was judged according to the intensity of red and green fluorescence (Fig. 1B, C). The efficiency of the transfection of the overexpression and interference *LEF-1* vectors was then assessed via quantitative real-time PCR. Cells transfected with no plasmid were included as a negative control. As shown in Fig. 3, the *LEF-1* expression level in the primary DPCs was 9.25-fold and 0.2-fold that of the control in the overexpression and interference groups, respectively (*p* < 0.001). Similar results were detected in the secondary DPCs, with *LEF-1* expression levels in the overexpression group and interference group being 10.53-fold and 0.21-fold, respectively, that of the control (*p* < 0.001). These results confirmed that overexpression and interference of *LEF-1* in the Arbas White Cashmere goat DPCs were successfully achieved.

*LEF-1* activated the expression of the *cyclin D1* and *C-myc* genes in the primary and secondary dermal papilla cells

Real-time PCR showed the consistent expression patterns of *LEF-1* and a series of
genes related to hair follicle growth in the primary and secondary DPCs. Compared with the negative control group, the mRNA expression levels of *cyclin D1*, *C-myc*, and *β-catenin* were increased and decreased in the *LEF-1*-overexpression and interference groups, respectively, to different extents (Fig. 3). Furthermore, western blot results showed the same patterns in protein expression levels (Fig. 4). Therefore, our results show that the expression levels of *cyclin D1*, *C-myc*, and *β-catenin* are positively correlated with *LEF-1* expression. Comparison of the gene expression levels between the primary and secondary DPCs of the control group showed that *LEF-1*, *β-catenin*, and *cyclin D1* mRNA expression levels in the secondary DPCs were 1.28-, 2.19-, and 1.16-fold higher than those of the primary DPCs, respectively. By contrast, the mRNA expression level of *C-myc* in the secondary DPCs was 0.37-fold that of the primary DPCs (Fig. 5).

**Effect of LEF-1 on the proliferation of dermal papilla cells**

Analysis of the growth curve of the different cell lines showed that *LEF-1* overexpression significantly increased the cell proliferation rate, especially as of the third day, whereas the growth of the *LEF-1* interference group was slower than that of the control group (Fig. 6).

We also investigated the function of *LEF-1* in promoting DPC proliferation via the EdU incorporation assay. We obtained similar results in primary dermal papilla and secondary DPCs (Fig. 7, 8). When *LEF-1* was overexpressed, EdU-positive cells were significantly increased relative to the control group. In primary and secondary DPCs,
the proportion of EdU-positive cells increased by more than 30%. EdU-positive cells were significantly reduced relative to the control group when $LEF-1$ was lowly expressed. In both primary and secondary DPCs, the percentage of EdU-positive cells is reduced by about 26%.

These results showed that overexpression of $LEF-1$ promoted the proliferation of DPCs, while downregulation of $LEF-1$ decreased the proliferation of DPCs.

**DISCUSSION**

The hair follicle is a complex sub-organ structure that continuously cycles through growth, degenerative, and telogen phases for continuous hair renewal. Dermal papillae interact with the cells of the bulge region toward the end of the telogen phase. By activating the proliferation of stem cells, dermal papillae are pushed down and the various hair follicle cells are differentiated to form new hair follicles (Kulessa et al. 2000; Stenn and Paus 2001). Therefore, DPCs are considered to be the center of hair growth regulation. To improve cashmere quality from goat breeds, it is necessary to elucidate the regulatory mechanisms underlying the proliferative effects if DPCs. Therefore, this study was designed to investigate the role of $LEF-1$ in the regulation of DPC proliferation via the Wnt signaling pathway.

Our experimental results showed that $LEF-1$, an important member of the Wnt signaling pathway, is involved in the regulation of DPC proliferation. With the increase of $LEF-1$ expression, DPCs proliferation increased. Moreover, when the expression of $LEF-1$ was knocked down, the proliferation ability of DPCs was decreased. The Wnt signaling pathway is closely associated with hair follicle growth and development, as
well as cell cycle maintenance (Andl et al. 2002; Huelsken et al. 2001). Our results also support this conclusion. In addition, we found that LEF-1 expression was correlated with β-catenin expression. Some studies have reported that in mouse embryonic day 11 (E11), LEF-1 was expressed in the mesenchyme of the mouse tentacle (Liu et al. 2004). During hair follicle development, LEF-1 levels in mesenchymal cells gradually increased with the formation of the basal lamina, and appeared in the embryonic epithelial cells. The TOP β-galactosidase (TOPGAL) activity of the Wnt reporter gene was localized in the positive region of LEF-1 expression. At E16, the expression of LEF-1 began to weaken in the epithelium and was concentrated in the hair matrix of the hair bulb, hair shaft, and DPCs of mesenchymal origin. TOPGAL was expressed in both the hair matrix and hair shaft (Liu et al. 2004; Zhou et al. 1995). These results show that LEF-1 and β-catenin have similar expression patterns in the development of hair follicles. Therefore, we hypothesize that the simultaneous changes of LEF-1 and β-catenin expression alter the expression of downstream target genes in the Wnt signaling pathway, thereby affecting the proliferation of DPCs.

As two target genes of the Wnt signaling pathway, cyclin D1 and C-myc are critical genes involved in cell proliferation and differentiation (Pinto and Clevers 2005). C-myc is an oncogenic transcription factor that plays an important role in the regulation of cell proliferation, differentiation, and aging (Dang et al. 1999). The high expression of C-myc positively regulates the activity of cyclin-dependent kinases (CDKs), which in turn accelerates the progression of G1 phase; this cascade is crucial for cell proliferation. Changes of C-myc expression mediated by the Wnt/β-catenin signaling pathway have
been reported in several cell types, and these changes affected the cell proliferation rate (Myant and Sansom 2011). In the present study, with the high expression of LEF-1, the expression of C-myc in dermal papilla cells also increased, and the proliferation rate of the cells increased. This suggests that the Wnt/β-catenin signaling pathway induces proliferation in dermal papilla cells via C-myc. However, C-myc is not the only target gene that regulates the proliferation of dermal papilla cells in the Wnt/β-catenin signaling pathway; cyclin D1 is also a target gene in the signaling pathway. Cyclin D1 is an important cyclin protein that regulates cell proliferation from the G1 phase to the S phase by mediating the functions of CDK4 and CDK6. In this study, we observed that LEF-1 promoted the proliferation of dermal papilla cells and also detected corresponding changes in cyclin D1 levels. This indicates that the proliferation of dermal papilla cells can be induced through the target gene cyclin D1 in the Wnt signaling pathway.

In this study, the expression levels of LEF-1 and β-catenin in primary dermal papilla cells were lower than those in secondary dermal papilla cells, and we also found that C-myc was more prominent in primary dermal papilla cells. It is speculated that the secondary dermal papilla cells may have stronger Wnt/β-catenin/LEF-1 signaling ability than primary dermal papilla cells, whereas signal-stabilized β-catenin can form complexes with various binding proteins and activate the downstream genes. These signal pathways are modified in different types of dermal papilla cells at different intensities, which may affect the direction of the development of primary and secondary hair follicles.
In summary, our experiments demonstrated that the overexpression of LEF-1 in dermal papilla cells upregulated the expression of C-myc and cyclin D1, and consequently promoted the proliferation of dermal papilla cells. Similarly, the interference of LEF-1 in dermal papilla cells reduced the expression levels of the C-myc and cyclin D1 genes, thereby decreasing the proliferation of dermal papilla cells. Therefore, a positive correlation between LEF-1 and the C-myc and cyclin D1 genes in dermal papilla cell proliferation was established. This is consistent with the classic Wnt signal pathway regulation characteristics. Our results also suggest that different types of Wnt signal modification will result in the formation of different hair follicle types, and provide the basis for future research on the molecular mechanism of hair follicle growth and development.

ACKNOWLEDGEMENTS

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REFERENCES


Fig. 1. Plasmids were transfected into Cashmere goat DPCs. (A) No plasmid transfected DPCs (B) DPCs transfected with the LEF-1-overexpressing vector. (C) DPCs transfected with the LEF-1-interference vector. Scale bar = 100 μm. DPC, dermal papilla cells; PHF, primary hair follicle; SHF, secondary hair follicle.

Fig. 2. Identification of Cashmere goat DPCs. The expression of α-SMA and CD133 was detected by using fluorescence microscopy; the nuclei were stained with DAPI. Scale bar = 100 μm.

Fig. 3. LEF-1 activated the expression of the cyclin D1, C-myc, and β-catenin genes in DPCs. The mRNA expression levels of LEF-1, cyclin D1, C-myc, and β-catenin were detected with quantitative real-time PCR. Green, No plasmid transfected DPCs; red, DPCs transfected with the LEF-1-overexpressing vector; yellow, DPCs transfected with the LEF-1-interference vector. Data are the means ± SD from three independent experiments. **P < 0.01; ***P < 0.001; ****P < 0.0001.

Fig. 4. Western blot. Lane 1, No plasmid transfected DPCs; Lane 2, DPCs transfected with the LEF-1-overexpressing vector; Lane 3, DPCs transfected with the LEF-1-interference vector.

Fig. 5. The mRNA expression levels of LEF-1, β-catenin, cyclin D1, and C-myc genes in primary (blue) and secondary (purple) DPCs. Data are means ± SD from three independent experiments. *P < 0.05; ****P < 0.0001.

Fig. 6. The growth curves of DPCs. Green, No plasmid transfected DPCs; red, DPCs transfected with the LEF-1-overexpressing vector; yellow, DPCs transfected with the LEF-1-interference vector.

Fig. 7. EdU incorporation assay. Proliferating cells were stained with EdU (red); the nuclei were stained with Hoechst 33342 (blue). Scale bar = 100 μm.

Fig. 8. The EdU incorporation rate was expressed as the ratio of EdU-positive cells to total Hoechst 33342-positive cells. Green, No plasmid transfected DPCs; red, DPCs transfected with the LEF-1-overexpressing vector; yellow, DPCs transfected with the LEF-1-interference vector. Data are the means ± SD from three independent experiments. **P < 0.01; ***P < 0.001.
Table 1. Primers used for real-time PCR.

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Fig. 1. Plasmids were transfected into Cashmere goat DPCs.

120x142mm (220 x 220 DPI)
Fig. 2. Identification of Cashmere goat DPCs.

147x106mm (220 x 220 DPI)
Fig. 3. LEF-1 activated the expression of the cyclin D1, C-myc, and β-catenin genes in DPCs.
Fig. 4. Western blot.

150x100mm (100 x 100 DPI)
Fig. 5. The mRNA expression levels of LEF-1, β-catenin, cyclin D1, and C-myc genes in primary (blue) and secondary (purple) DPCs.

146x90mm (220 x 220 DPI)
Fig. 6. The growth curves of DPCs.

146x49mm (220 x 220 DPI)
Fig. 7. EdU incorporation assay.

146x85mm (220 x 220 DPI)
Fig. 8. The EdU incorporation rate was expressed as the ratio of EdU-positive cells to total Hoechst 33342-positive cells.