LNCRNA2919 MEDIATED HAIR FOLLICLE DEVELOPMENT AND GROWTH IN ANGORA RABBITS

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ABSTRACT

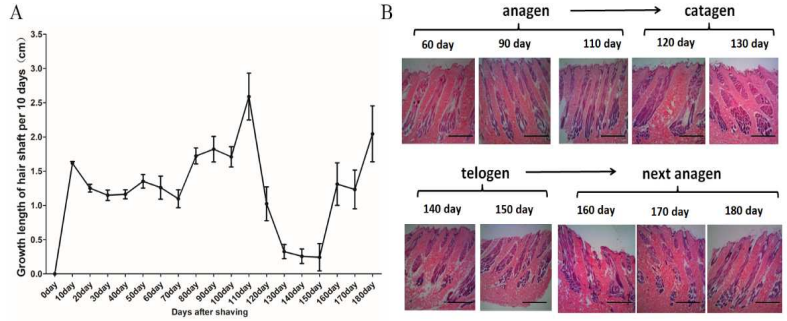
The periodic regeneration of hair follicle plays a critical role in wool production. Therefore, it is an effective way to carry out the research on periodic regeneration of hair follicle to increase wools production. In this study, whole-transcriptome analysis was performed to investigate ncRNAs and mRNAs associated with the various stages of the HF cycle in Angora Rabbits. The full length, intracellular localization and coding ability of key factor lncRNA2919 were identified. Through the test of adenovirus system infection in vitro and vivo, it was found that lncRNA2919 could inhibit the periodic regeneration of rabbit hair follicles. Further, the binding proteins of lncRNA2919 were screened by RNA pull-down and mass-spectrometric, such as STAT1, KRT16 and so on. The results will fill the gap in the research field of hair follicle regeneration from the level of long non-coding RNA, and provide a new idea to accurate breeding and high-quality production in wool traits of Angora rabbit.

Key words: Hair follicle development, Angora rabbits, lncRNA2919, STAT1

INTRODUCTION

The hair follicle (HF) cycle is a complicated and dynamic process in rabbits, associated with various signaling pathways and gene expression patterns(Hardy, 1992;Aubin-Houzelstein, 2012;Harel et al., 2015;Si et al., 2018). Most non-coding RNAs (ncRNAs) are RNA molecules that are not translated into proteins but are involved in the regulation of various cellular and biological processes(Yue et al., 2016;Wang et al., 2017).

Figure 1: Hair follicle synchronization model in Angora rabbits.



(A) Measurement of the hair coat length of Angora rabbits. (B) HE staining of

In this study, we explored the relationship between ncRNAs and the HF cycle by developing a synchronization model in Angora rabbits. Transcriptome analysis was performed to investigate ncRNAs and mRNAs associated with the various stages of the HF cycle. 107 long non-coding RNAs (lncRNAs), 247 circular RNAs (circRNAs), 97 microRNAs (miRNAs), and 1,168 mRNAs were differentially expressed during the three HF growth stages (Zhao et al., 2019)(Figure 1). Among them, lncRNA2919 was highly expressed in the catagen and telogen, suggesting that it might play important roles in rabbit hair follicle development.

In the field of hair follicle development, the research of lncRNAs is very limited, and its specific mechanism still needs in-depth study. In this study, we identified lncRNA2919, verified the function of lncRNA2919 in the development of hair follicles in vivo, and further explored the downstream signaling

molecules of lncRNA2919. The research aims to clarify the development mechanism of rabbit hair follicles from the perspective of lncRNAs, provide new ideas for precise breeding and efficient production of Angora rabbits.

MATERIALS AND METHODS

Analysis of the full-length sequence and coding ability of lncRNA2919

According to the full length sequence of lncRNA2919 predicted by the whole transcriptome lncRNA-seq (Zhao et al., 2019), the full length sequence of lncRNA2919 was amplified by 5' and 3' RACE experiments. Analysis of the possible open reading frame and potential coding ability of lncRNA2919 was performed by Online Analysis Website ORF (Open Reading Frame) finder (https://www.ncbi.nlm.nih.gov/orffinder/) and CPC2 (Coding Potential Calculator 2) (http://cpc2.cbi.pku.edu.cn).

The coding ability of lncRNA2919 was further verified by the tag vector. One-step cloning primers for pcDNA3.1-3×Flag tag vector were designed using CE Design V1.04, and the start codon ATG was inserted at the 5' end of lncRNA2919, and T and TT bases were added to the 3' end of the pcDNA3.1-Flag-lncRNA2919-T and pcDNA3.1-Flag-TT product, respectively. After transfecting the cells, use the Western bolt to detect the protein expression level.

Subcutaneous injection of lncRNA2919 adenovirus *in vivo*

The rosin and paraffin were heated and mixed in the ratio of 1:1, the mixture was applied to the back of long-haired rabbits after cooling, and part of the back coat was plucked, the skin showed a slight red color after plucking, indicating that the hair follicle structure was destroyed and the hair follicle cycle was contemporaneous to the next growth period. Subcutaneous injection experiments were performed and divided into three groups (four biological replicates per group), including overexpression (OE) group (50 μL AD-OE-lncRNA2919+150 μL saline), knockdown (KD) group(50 μL AD-KD-lncRNA2919+150 μL saline) and saline control group (200 μL saline).

Diluted adenovirus solution or saline was injected into the skin of the back plucked rabbits using a syringe. After subcutaneous injection, the solution appears as a small mound on the skin surface and were gradually absorbed over time. Photographs were taken at regular intervals to observe the condition of the rabbits after adenovirus injection. Seven days after the first injection, the adenovirus was injected subcutaneously again, and 7 days after the second injection, the back skin was collected for sectioning and RNA extraction experiments.

Analysis of RNA pull-down and mass spectrometry

Based on the obtained full-length sequence of lncRNA2919, PCR primers of the sense strand and antisense strand were designed, and the sense and antisense strands of lncRNA2919 were obtained by PCR amplification. In vitro transcription was performed using the in vitro transcription kit. In vitro transcribed RNA was extracted and RNA pull-down assays were performed using the RNA pull-down kit. After the target RNA was labeled with a probe, it was combined with the magnetic beads to elute the RNA-binding protein complex, and the eluate was collected.

The target proteins were separated by vertical electrophoresis on PAGE gel, and the PAGE gel was silver-stained to observe the protein bands. The target bands were cut from the gel and the proteins were identified using mass spectrometry. The proteins were subjected to reductive alkylation and enzymatic digestion, and the proteolyzed peptides were desalted after enzymatic digestion. The proteolyzed peptides were analyzed by LC-MS/MS instrument on the machine, and the raw data were submitted directly to the Proteinpilot software connected to the AB SCIEX Triple TOF™ 5600 plus mass spectrometer for database search. Conf $\geq$ 95% and Unique peptides $\geq$ 1 were set to analyze the peptides and proteins identified in the samples. The information related to the mass spectra of the identified proteins is analyzed to obtain the final protein identification results.

RESULTS AND DISCUSSION

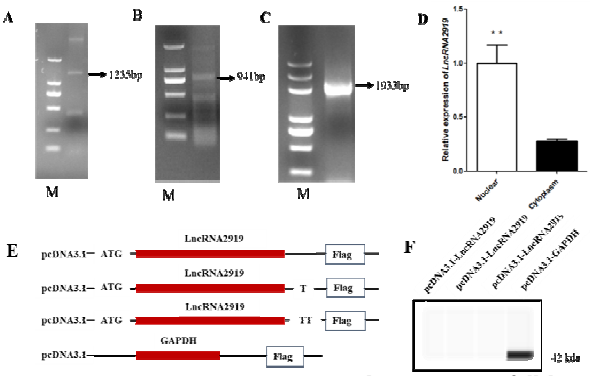
Analysis of the full-length sequence and coding ability of lncRNA2919

In general, lncRNA is less conserved in different species. The mechanism of lncRNA in the rabbit hair follicles development and growth is still unclear. Therefore, we obtained a 1235-bp 5'UTR and a 941-bp

3'UTR of lncRNA2919 by 5' RACE and 3' RACE, respectively (Figure 2). Then, the sequences were spliced and aligned to the rabbit genome, and finally lncRNA2919 was located at chromosome 161145434-161147366 of rabbit genome, with a total length of 1933 bp.

Further, it was confirmed that lncRNA2919 was mainly expressed in the nucleus by nuclear separation and qRT-PCR experiments. Analyzing the possible ORFs of lncRNA2919, it is found that although lncRNA2919 can predict 14 ORFs, they are all less than the number of amino acids of the normal coding gene (100aa), suggesting that lncRNA2919 is non-coding RNA. Using CPC to predict the coding ability of lncRNA2919, the score is -0.575049, suggesting it is a non-coding RNA.And the pcDNA3.1-Flag vectors of LncRNA2919 and its fragments were constructed, it was found that lncRNA2919 had no protein coding ability by Western blot.

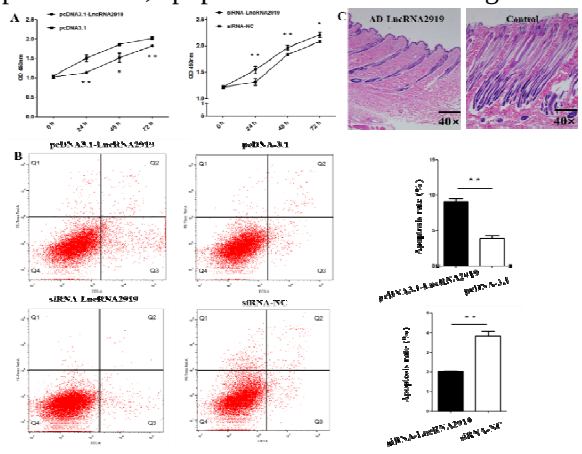
Figure 2: Identification, intracellular localization and protein coding of lncRNA2919.



(A) 5' RACE. (B) 3' RACE. (C) lncRNA2919 full-length sequence. (D) lncRNA2919 intracellular localization. (E) Constructions of pcDNA3.1-Flag vectors of lncRNA2919 and its fragments. (F) Western blot analysis of lncRNA2919 constructions, GAPDH as a control.

Effect of adenovirus-mediated lncRNA2919 on hair follicle regeneration in Angora rabbits

Figure 3: Effects of lncRNA2919 on DPC proliferation, apoptosis and hair follicle regeneration.



(A)lncRNA2919 inhibited DPC proliferation. (B) lncRNA2919 promoted DPC apoptosis. (C) Tissue sections after *in vitro* subcutaneous injection of AD-lncRNA2919.

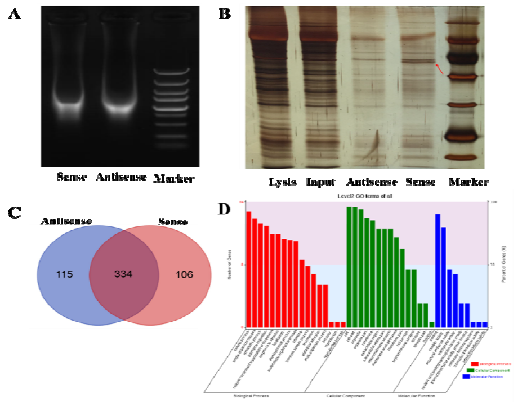
In order to study the biological function of lncRNA2919 in hair follicle development, lncRNA2919 overexpression and interference were constructed in dermal papilla cells (DPC). By flow cytometry, lncRNA2919 was found to inhibit DPC proliferation and promote apoptosis ($P<0.05$). In vitro, the AD-lncRNA2919 adenovirus overexpression system was prepared and injected by subcutaneous injection. Using tissue sectioning method, it was found that the number of hair follicles in lncRNA2919-injected group were significantly lower than that in the control group, and the density and depth of hair follicles were significantly reduced. It shows that lncRNA2919 can significantly inhibit the regeneration process of hair follicles and delay the hair follicles into the next growth cycle (Figure 3). And AD-OE-lncRNA2919 can significantly increase the expression level of lncRNA2919 in the Angora rabbit skin ($P<0.01$), and significantly upregulate *FGF5* and *KRTAP11-1*, and *STAT1* ($P<0.01$), extremely significantly down-regulated the expression of *LEF1* and *WNT2*, *BCL2* and *CCND1* ($P<0.05$). AD-KD-lncRNA2919 has the opposite result.

RNA pull-down combined with mass spectrometry screening for lncRNA2919 interacting proteins

To determine the lncRNA2919-mediated signal transduction pathway, we obtained the RNA by in vitro transcription and the proteins that interact with lncRNA2919 using the RNA pull down technique (Figure 4). By SDS-PAGE silver staining and mass spectrometry, the differentially proteins were identified.

lnc002919_antisense and lnc002919_sense identified 555 proteins, of which 334 proteins were both identified. lnc002919_sense identified 106 unique proteins. The proteins identified in lncRNA2919_antisense and sense were analyzed by GO terms based on the mass spectrometry identification results obtained. The results showed that the unique proteins GO terms identified in lncRNA2919_sense were mostly enriched in cellular processes, bioregulation, metabolic processes, cellular components and molecular binding, from which the proteins related to hair follicle development such as hair follicle cycle development, epithelial cell morphogenesis and keratinization and other biological functions related to skin hair follicle development were screened, such as STAT1 (Calò et al., 2003), and KRT16 (Koster, 2012), *etc*.

Figure 4 : Screening of the interaction protein of lncRNA2919 using RNA pull down and protein mass spectrometry.



(A) in vitro transcription of lncRNA2919. (B) Silver staining of in vitro transcription-enriched protein. (C) Venn diagram analysis of the proteins identified by Lnc002919_antisense and lnc002919_sense. (D) Enrichment analysis of lncRNA2919_antisense-specific binding protein.

CONCLUSIONS

This research comprehensively identified candidate regulatory ncRNAs during the HF cycle by transcriptome analysis. The molecular mechanism of lncRNA2919 regulated the HF cyclic regeneration was revealed from in vitro to in vivo, and binding proteins of lncRNA2919 were screened to further analyze the downstream signaling transduction network of lncRNA2919. This study provides a basis for systematic further research and new insights on the regulation of HF cycle.

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