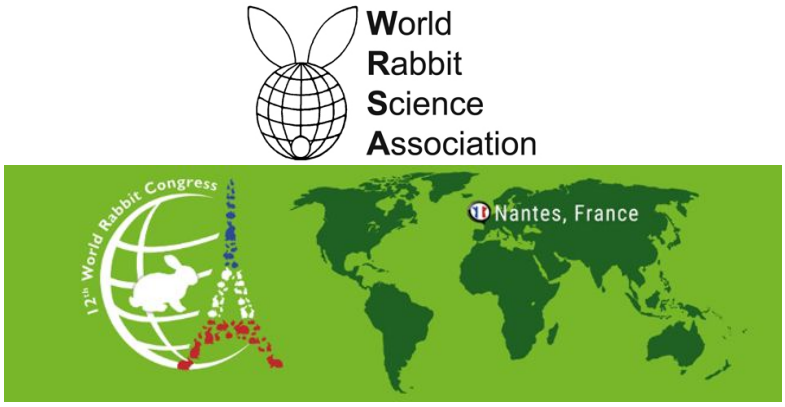

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ANALYSIS OF HISTOLOGICAL AND MICRO-RNA PROFILES CHANGES IN RABBIT SKIN DEVELOPMENT

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ABSTRACT

The periodic regrowth of rabbit fur is economically important. Here, we aimed to characterise the histological traits and micro-RNA (miRNA) expression profiles in the skin of Wan Strain Angora rabbits at different weeks after plucking. Haematoxylin-eosin staining showed that hair follicles were in the telogen phase in the first week, while they were in the anagen phase from the fourth to twenty-fourth weeks. In addition, two small RNA libraries were constructed. 185 miRNAs were differentially expressed between the telogen and anagen phases. The function of the differentially expressed miRNAs was explored by comparing them with known mammalian miRNAs and by GO and KEGG analysis of their predicted targets. The fibroblast growth factor 5 (*FGF5*) gene was verified to be a target of conservative_NC_013672.1_9290 and conservative_NC_013675.1_10734. We investigated differential miRNA profiles between the telogen and anagen phases of rabbts hair cycle and preliminarily revealed the miRNA-mediated regulation of rabbits hair follicle cycling.

Key words: Rabbit, hair follicle cycling, miRNA, FGF5.

INTRODUCTION

The growth of hair follicles are cyclical (anagen, catagen and telogen) throughout a rabbit's life. Each growth period has a specific activated/silenced gene expression pattern (Liu et al., 2018). miRNA/mRNA regulatory networks are reported to be involved in the regulation of hair follicle development and epidermal homeostasis (Botchkareva, 2012). miRNAs are also involved in the regulation of signalling pathways and factors related to skin development and hair cycle(Luan et al., 2017; Fu et al., 2014). Although substantial progress has been made in discovering important regulators of these processes in humans and animal (Wagner et al., 2018; Zhang et al.,2017) few studies have focused on the hair follicle cycle in rabbits. In the present study, we investigated the hair cycle and its related miRNAs in rabbits, so as to further understand the regulatory role of miRNA in the hair growth cycle.

MATERIALS AND METHODS

Animals

The rabbits were procured from the rabbit farm of the Institute of Animal Husbandry and Veterinary Medicine of Anhui Academy of Agriculture Sciences, Hefei, Anhui, China, raised and managed under the same conditions, feeding pellets restrictedly and drinking water *ad libitum*.

Sample collection, preparation, and histological examination

Skin tissue samples (about 1 cm^2) from the back of each rabbit were collected in the first, fourth, eighth, and twenty-fourth weeks after plucking hairs and cutted in two pieces. One piece were fixed in 4% paraformaldehyde solution for histological analysis and were washed with running water, dehydrated

using an ethyl alcohol series, cleaned in xylene, and embedded in paraffin wax. The specimens were sectioned to a thickness of 4 μm using a Leica RM2235 microtome. Samples of transverse and vertical cross-sections were stained with haematoxylin-eosin, examined, and photographed using an Olympus BX51 biomicroscope.

Small RNA library construction, sequencing and analysis

Skin samples were isolated from the dorsal skin of three Wan Strain Angora rabbits in the first week after hair plucking (S01 samples), when the wool cycle is expected to be in the telogen phase, and in the eighth week post-plucking (S02 samples), i.e., in the anagen phase. Total RNA was extracted using TRIzol reagent. Two small RNA (sRNA) libraries were constructed and generated using the Next Ultra small RNA Sample Library Prep Kit (Illumina). The novel mature miRNAs were blasted to miRNAs of other mammals (humans, mice, pigs, cows, rats, and sheep) using miRBase 21.0. miRNAs in the libraries with identical or related sequences (0 nucleotide substitutions and vacancy permitted) to other mammals were identified as known mature miRNAs. The miRNA expression levels were estimated by TPM (transcript per million) values. $|\log2 \text{ (Fold Change)}| \geq 1$ and FDR ≤ 0.01 were set as the default threshold for significantly differential expression. RNAhybrid and miRanda were used to predict the genes targeted miRNA. GO and KEGG enrichment analyses were performed on miRNA targets.

Statistical analyses

Student's *t*-test was used for statistical comparisons. The results with a *P* value < 0.05 were considered as indicative of statistically significant differences.

RESULTS AND DISCUSSION

Characterization of the hair cycle

A morphological analysis showed an atrophic hair follicle structure in the first week (Fig. 1a, b). In contrast, a complete hair follicle structure and an obviously increased number of hair follicles were observed in the fourth, eighth, and twenty-fourth weeks (Fig. 1c–h). Moreover, the length of hair follicles in the first week was evidently shorter than those in the fourth, eighth, and twenty-fourth weeks (Fig. 1b, d, f, h). Similar lengths of hair follicles between the fourth, eighth, and twenty-fourth weeks were observed (Fig. 1d, f, h). The results showed that rabbit hairs grew in a linear growth rhythm from the fourth to twenty-fourth weeks for different types of hair, suggesting that hair follicles were still in the anagen phase until the twenty-fourth week after plucking.

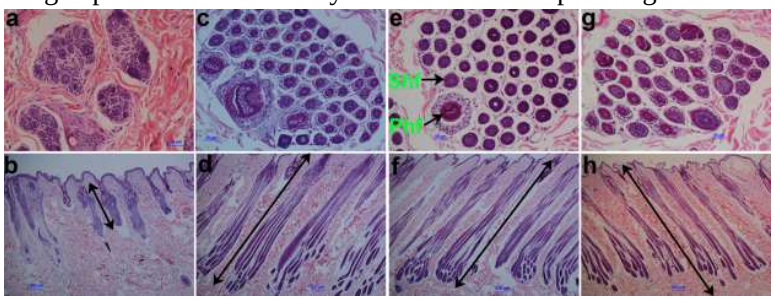

Figure 1: Skin histology at different weeks after hair plucking. (a), (b): the 1st week. (c), (d): the 4th week. (e), (f): the 8th week. (g), (h): the 24th week. Phf, Primary hair follicles, Shf, Secondary hair follicles

miRNA profiles of skin tissues in the telogen and anagen stages of hair follicles

We characterised the miRNA profiles in the telogen and anagen stages of hair follicles in Wan Strain Angora rabbits. Since the Rfam and GenBank databases contain few known rabbit miRNAs, the novel miRNAs were compared with known mammalian miRNAs (mature miRNAs) in the miRBase 21.0 database to expand the subset of "known" miRNAs in rabbit skin. 43 DE rabbit miRNAs were found to be conserved among various species (Table 1).

Table 1 The number of known mature miRNAs compared with known mammalian miRNAs.

Sample	Cow	Human	Mouse	Pig	Rat	Sheep	Total	DE miRNAs
S01	260	265	231	208	226	88	242	43
S02	263	264	232	208	226	88	242	43

185 miRNAs with significantly different expression between the telogen and anagen stages were detected. Among them, there were 43 known mature miRNAs. The results demonstrated the different roles of DE miRNAs in regulating the hair cycle and revealed a large number of novel miRNAs controlling the hair cycle in Wan Strain Angora rabbits.

miRNA target gene prediction, verification, and functional annotation analyses

FGF5 was predicted as the common target gene of the conservative_NC_013672.1_9290 (miRNA 1) and conservative_NC_013675.1_10734 (miRNA 2). q-PCR analyses revealed that *FGF5* gene was a target of the two miRNAs (Fig. 2). *FGF5* serves as a crucial regulator in hair length (Higgins et al., 2014) and influences the hair cycle by regulating the anagen–catagen transition (Akilli et al., 2015). Our results indicated that miRNA 1 and miRNA 2 are candidate regulatory miRNAs in the hair cycle.

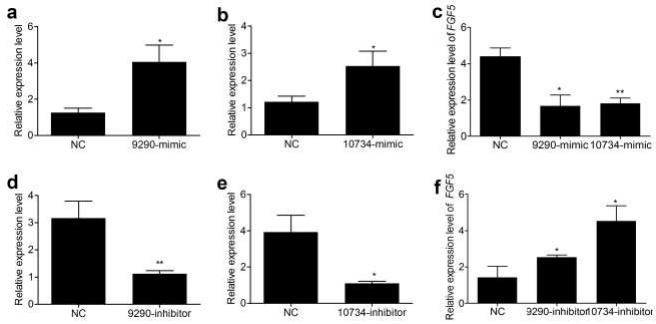

Figure 2: Identification of FGF5 as a target of miRNA 1 and miRNA 2 in RAB-9 cells. (a),(b): relative expression of miRNA 1 and miRNA 2 after transfecting mimics, respectively. (d),(e): relative expression of miRNA 1 and miRNA 2 after transfecting inhibitors, respectively. (c),(f): relative expression of endogenous FGF5 mRNA after transfecting miRNA 1 and miRNA 2 mimics and inhibitors, respectively.

In addition, GO term annotation results showed that hair follicle development, hair cycle, and lipid catabolism were significantly enriched by the targets of DE miRNAs (Fig. 3), suggesting the DE miRNAs were potential regulators of hair follicle development and lipid metabolism in rabbits. KEGG analysis showed that Wnt signalling, TGF-β signalling, ECM-receptor interactions, apoptosis, as well as fat digestion and absorption pathways, were enriched by targets of DE miRNAs, suggesting their potential involvement in the regulation of hair follicle cycling in rabbits. Wnt signalling is a key regulator of hair follicle morphogenesis, life-long hair follicle regeneration (Niemann et al., 2014) and cell signals conveying in skin (Zhao et al ., 2017). ECM-receptor interactions are essential for the morphogenesis of hair follicles and potentially regulate hair growth (Ding et al., 2019)

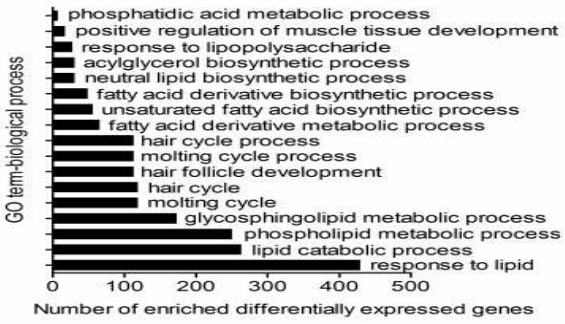

Figure 3: Significantly enriched GO terms for target genes of DE miRNAs between telogen and anagen stages (P<0.05). The x-axis represents the number of target genes; the y-axis represents the GO terms.

Our observation that a large proportion of target genes of newly identified DE miRNAs were implicated in fatty acid metabolism was in line with the previous study reporting that lipid metabolism is a major determinant of wool diameter and hair growth (Fu et al., 2016).

CONCLUSIONS

This study has identified differences in hair follicle histology and miRNA profiles between the telogen and anagen phases in rabbit. New miRNAs, potentially involved in the rabbit hair cycle, were revealed. This study serves as the basis for functional studies addressing the role of miRNAs in the rabbit hair cycle.

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