World Rabbit Science

World Rabbit Sci. 2018, 26: 179-190
doi:10.4995/wrs.2018.7058

IDENTIFICATION AND PROFILING OF MICRO-RNA BETWEEN BACK AND BELLY SKIN IN REX RABBITS (*ORYCTOLAGUS CUNICULUS*)

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Abstract: Skin is an important trait for Rex rabbits and skin development is influenced by many processes, including hair follicle cycling, keratinocyte differentiation and formation of coat colour and skin morphogenesis. We identified differentially expressed microRNAs (miRNAs) between the back and belly skin in Rex rabbits. In total, 211 miRNAs (90 upregulated miRNAs and 121 downregulated miRNAs) were identified with a $|\log_2$ (fold change)|>1 and *P*-value<0.05. Using target gene prediction for the miRNAs, differentially expressed predicted target genes were identified and the functional enrichment and signalling pathways of these target genes were processed to reveal their biological functions. A number of differentially expressed miRNAs were found to be involved in regulation of the cell cycle, skin epithelium differentiation, keratinocyte proliferation, hair follicle development and melanogenesis. In addition, target genes regulated by miRNAs play key roles in the activities of the Hedgehog signalling pathway, Wnt signalling pathway, Osteoclast differentiation and MAPK pathway, revealing mechanisms of skin development. Nine candidate miRNAs and 5 predicted target genes were selected for verification of their expression by quantitative reverse transcription polymerase chain reaction. A regulation network of miRNA and their target genes was constructed by analysing the GO enrichment and signalling pathways. Further studies should be carried out to validate the regulatory relationships between candidate miRNAs and their target genes.

Key Words: Rex rabbit, back skin, belly skin, miRNA, transcriptome.

INTRODUCTION

By targeting mRNAs, microRNAs (miRNAs), which are short non-coding RNAs, play crucial roles in plants and animals (Bartel, 2004; Callis *et al.,* 2007). Hundreds of miRNAs have been obtained from nearly all metazoan genomes, including worms, plants and mammals (Naeem *et al.,* 2010). As hidden messages in genomes, miRNAs have diverse expression patterns and regulate various developmental and biological processes; miRNAs provide information to understand the mechanism of cellular proliferation and differentiation, as well as the occurrence of diseases (Alexander *et al.,* 2013; Kochegarov *et al.,* 2013; Xia *et al.,* 2013; Kureel *et al.,* 2014). While miRNAs are widely expressed in a variety of species, miRNA expression patterns are specific to different tissues, developmental stages, and individuals (Bashirullah *et al.,* 2003; Neilson *et al.,* 2007). Skin is the biggest organ in animals and skin development is a complex dynamic process, which involves development of the epidermis, hair follicle (HF) cycling, hair morphogenesis and the formation of skin colour (Schneider, 2012; Tian *et al.,* 2012; Ahmed *et al.,* 2014). To date, many studies have focused on the role of miRNAs in the regulation of skin development. For example, the downregulation of miR-34a and miR-34c significantly restored epidermal cell proliferation (Antonini *et al.,* 2010). MiR-214 plays a key role in the Wnt signalling pathway that regulates skin morphogenesis and the development of HFs (Ahmed *et al.,* 2014). Furthermore, miR-205 is highly expressed in the epidermis, and acts as a regulator of cell adhesion and migration. The inhibition of miR-205 induces the cytoskeleton to rebuild and miR-205 especially regulates the migration of

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https://doi.org/10.4995/wrs.2018.7058

keratinocytes (Yu *et al.,* 2008, 2010). Many miRNAs play roles in regulating skin colour formation. MiR-204/211 targets MITF to regulate pigmentation, and differentially expressed miRNAs have been found in MITF knock-down melanocytes (Adijanto *et al.,* 2012; Wang *et al.,* 2012).

Rex rabbits are famous for their fur, which is soft and light. Many studies of skin development in rabbits have been conducted. Transcriptomic analysis of plaice and un-plaice Rex rabbits have revealed the key genes and SNPs involved in fur development (Pan *et al.,* 2015). MLPH is a crucial gene for the dilute locus in rabbits and polymorphisms of this gene lead to different coat colours (Fontanesi *et al.,* 2014). KIT is closely related to the English spotting coat colour locus (Fontanesi *et al.,* 2014). However, there has been little focus on the regulation of miRNAs for skin development on different parts of rabbits, as studies on the miRNA transcriptome involved in skin development are scarce in rabbits.

In this study, Chinchilla Rex rabbits were selected, due to their unique differences in fur between the back and belly and an analysis of differentially expressed miRNAs between the back and belly was performed. Differentially expressed miRNAs with target genes related to HF cycling, hair morphogenesis, epidermis formation and coat colour were analysed. The results obtained improve our understanding of skin development in rabbits and provide a valuable theoretical basis for further miRNA research.

MATERIALS AND METHODS

Tissue collection

The 3 Rex rabbits used in this experiment were obtained from Zhejiang province (Yuyao Xinnong Breeding Co., Ltd). The Rex rabbits were from the same litter and were raised by their mother for 20 d. The fur of the Chinchilla Rex rabbits on their back was dark blue at the stem base, pearl grey in the middle and black at the burr point, while fur on the belly was pale blue at the base and white at the tip (Figure 1), the skin cycling stages of our samples were the same, and the histologic sections of skin for samples are shown in Figure S1. Sodium pentobarbital solution (0.7%) was injected into the ear vein of the rabbits and we made sure rabbits were anaesthetised when the samples were taken. To prevent bacterial infection, the resulting lesion was smeared by iodine solution. Skin tissue samples of 1 cm^2 in size were taken from the backs (B group) and bellies (F group) of the 3 rabbits. Samples were immediately frozen in liquid nitrogen and stored at –70°C. All animal experiments were maintained according to recommendations proposed by the European Commission (1997) and all efforts were made to minimise suffering of the animals. Sample collection was approved by the Institutional Animal Care and Use Committee of the School of Animal Science and Technology at Yangzhou University and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988) and the Standards for the Administration of Experimental Practices (Jiangsu, China, 2008).

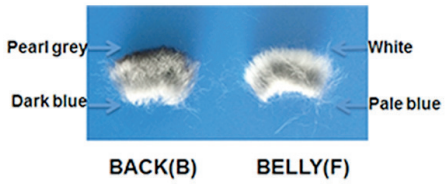

Figure 1: Fur from the back (left) and belly (right) of a Chinchilla Rex rabbit. The fur from the rabbit's back was dark blue at the base and pearl grey on top, whereas the fur on the belly was pale blue on the lower region and white on the upper region, and the length and diameter of the hair for Rex rabbits was different between the 2 parts.

Small RNA library construction and Illumina sequencing

Total RNA from six samples was extracted according to the manufacturer's protocol using the mirVana™ miRNA Isolation Kit. Total RNA was checked for quality and integrity using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US) with RIN (RNA Integrity Number) Library preparation. T4 RNA ligase was used to ligate 5' and 3' adapters to the small RNA molecules. Complementary DNA (cDNA) was obtained by reverse transcription of small RNAs ligated to 3' and 5' adapters using the SuperScript II Reverse Transcription Kit (Invitrogen, USA), and cDNA constructs were then amplified by PCR. An agarose gel was used to visualise the amplified cDNA constructs, and their quality and concentration was confirmed with an Agilent

2100 Bioanalyzer. Lastly, the purified cDNA libraries were quantified using a Qubit® 2.0 Fluorometer (Invitrogen, USA). Sample libraries completed cluster generation and first primer hybridisation on cBot, which matched the Illumina Hiseq 2500 platform. After cluster generation, the sequencing reagent was prepared according to the HiSeq 2500 User Guide, and samples were sequenced using single-end 1×50 nt multiplex reads. Sequencing was controlled by data collection software (Illumina, San Francisco, USA) and real-time image data was extracted and processed.

Analysis of miRNA high-throughput sequencing data

A chart showing the analysis process of miRNA sequencing data in Figure S2. Raw data were produced by Illumina sequencing and processed using fastx (fastx_toolkit-0.0.13.2). After filtering low-quality reads and trimming the adapter sequences, clean reads were obtained and were used to initially search against the databases. Reads were mapped to the genome by bowtie (v1.0.0) (Langmead and Salzberg, 2012) to analyse their expression and distribution on the genome, then reads were aligned to the miRBase 21.0 database (http://www.mirbase.org/) (Kozomara *et al.,* 2014) to identify known miRNAs, and related sequences (1~2 nucleotide substitutions permitted) were identified for miRNAs to *Oryctolagus cuniculus* or other mammals (*Homo sapiens*, *Mus musculus*, *Cricetulus griseus*, and *Rattus norvegicus*). Sequences that did not match to annotated sequences in the above databases were analysed by miRDeep2 (v.2.0.0.8) (Adamidi, 2008) to predict novel miRNAs. Based on the hairpin structure of the pre-miRNA and the miRBase database, the corresponding miRNA star sequence was also identified. The expression abundance of miRNAs in the 6 libraries was normalised to give the number of transcripts per million (TPM) using the following formula: Normalised expression=actual miRNA count/total count of clean reads ×1 000 000. Differentially expressed miRNAs between the 2 groups were identified using the DESeq algorithm (Anders, 2010) 002. in R (v3.2.0), and miRNAs were selected with $|\log_2$ (fold change)|>1 and *P* values <0.05.

The other reads which were not mapped to the genome were annotated and classified by alignment with reference non-coding RNAs from the Ensembl ncRNA (http://asia.ensembl.org/index.html), piRNA (http://pirnabank.ibab.ac.in/request.html) Rfam (v.10.1) (http://www.sanger.ac.uk/software/Rfam) and GenBank databases (http://www.ncbi.nlm.nih.gov/genbank/) by BLAST (v2.2.28+).

Predicted and analysis of target genes of differentially expressed miRNAs

Based on miRNA sequences, target genes of differentially expressed miRNAs were predicted by alignment of the gene sequences to rabbit genome cDNA sequences using Miranda (v3.3a) (Enright *et al.,* 2004) prediction software, with the following parameters: S≥150 ΔG≤−30 kcal/mol, strict 5' seed pairing. Functional annotation, classification and biological signalling pathway for target genes were identified using Ensembl, GO (Gene Ontology) and KEGG (Kyoto Encyclopaedia of Genes and Genomes). Target genes significantly enriched in GO functional annotation or KEGG pathways were identified using R (v3.2.0) based on the hypergeometric distribution.

qRT-PCR validation of miRNA data

To confirm the miRNA transcriptome data, 12 miRNAs were selected for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Approximately 6 µg total RNA was used to synthesise cDNA after adding a poly (A) tail to the end of the 3' end of the miRNAs using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen), according to the manufacturer's instructions. qRT-PCR was performed using miRcute miRNA qPCR Detection kit (SYBR Green). The specific primer sets are listed in Table S1. We chose the U6 small nuclear RNA gene as an internal control. Expression of miRNAs was defined based on the threshold cycle (Ct), and the results of the experiments were normalised to the expression levels of the constitutively expressed U6. Five candidate genes that were targets of differentially expressed miRNAs were selected for validation by qRT-PCR using the AceQ qPCR SYBR® Green Master Mix (Vazyme) according to the manufacturer's protocol. The specific sequences are listed in Table S2. We chose the rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. The results of the experiment were normalised to the expression level of the constitutive GAPDH gene. The quantitative variation was evaluated and the relative miRNA expression levels and target gene expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method. Error bars represent the mean±standard deviation and were calculated using GraphPad Prism 5. A paired t-test was performed to test for significance test between the 2 groups using SPSS 21.0.

Table 1: Summary of data quality and length distribution of Illumina sequencing reads.

Type	total reads	high quality	3'adapter null	insert null	5'adapter contaminants	smaller than 18nt	polyA	clean reads	clean ratio
B1	22,972,086	22,846,179	104,481	2,109	24,182	1,583,123	418	21,131,866	92.50%
B2	27,140,830	26,976,633	185,124	6,932	38,136	1,949,900	593	24,795,948	91.92%
B3	26,988,774	26,810,219	130,536	4,676	14,768	825,990	337	25,833,912	96.36%
F1	26,772,875	26,614,212	119,242	2,534	15,796	1,138,746	348	25,337,546	95.20%
F2	27,106,257	26,939,379	195,880	3,165	28,283	1,835,487	376	24,876,188	92.34%
F3	43,395,279	43,299,224	288,856	4,103	46,618	3,569,360	472	39,389,815	90.97%

RESULTS

Characteristics and sequence analysis of the small RNAs

Six small RNA libraries (B1, B2 and B3 group, F1, F2 and F3 group) were constructed from back and belly skin of the rabbits using Illumina sequencing technology. A total of 22,972,086 (B1), 27,140,830 (B2), 26,988,774 (B3), 26,772,875 (F1), 27,106,257 (F2), and 43,395,279 (F3) raw reads for each sample were generated, and after removing low quality reads, contaminants, adapters, poly-A sequences and reads which were shorter than 18 nucleotides, clean reads were obtained for further analyses (greater than 90% for each sample) (Table 1). All reads were deposited in the Short Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) with the accession number PRJNA395429.

The distribution of unique and total reads was counted and the distribution of sequences after filtering was analysed (Table 2). The distribution of length for the reads is shown in Figure S3; the majority of the reads are longer than 30nt in most libraries; these reads were mapped on tRNAs according to the pie charts in Figure S4, which showed the distribution of small RNAs among the various categories. The reads and small RNAs were also analysed for saturation (Figure S5); the saturation plot shows the amount of annotated, annotated small RNA and unique small reads were enhanced by the increasing of data size, which showed the sequencing of reads is efficient. High-quality small RNA reads were mapped to the genome of *Oryctolagus cuniculus* OryCun2.0. The numbers of total and unique sequences that matched the genome in the 6 libraries were analysed. All small RNA reads were classified into 1 of 13 annotation categories: scRNA, intron antisense, intron sense, srpRNA, snRNA, exon sense, exon antisense, unannotated, rRNA, snoRNA, repeat, tRNA, and miRNA.

Identification of potential novel miRNAs

In the miRbase 21.0, only 12 entries showing miRNA precursors and 21 entries for mature miRNA products from rabbits. To identify known miRNAs in rabbit, miRNA precursors and mature miRNAs of known mammalian miRNAs was compared with our dataset. A total of 4619 mature miRNAs were mapped to mature microRNAs in the miRBase 21.0 database. The hairpin structure of miRNA precursors was used to predict novel miRNAs and 101 potential novel miRNAs were identified (Table S3), which we named novel-miR-N (1~101) and we made a provisional identification

Table 2: Genome mapping statistics. Reads were mapped to the genome by bowtie to analyse their expression and distribution.

	Unique sRNA reads		Total sRNA reads	
	Total sRNA reads	Mapping to genome	Total sRNA reads	Mapping to genome
B1	1,043,494	367,633 (35.23%)	21,131,866	14,786,778 (69.97%)
B2	1,366,071	533,453 (39.05%)	24,795,948	18,122,875 (73.09%)
B3	942,865	369,127 (39.15%)	25,833,912	18,007,580 (69.71%)
F1	827,085	309,738 (37.45%)	25,337,546	17,376,158 (68.58%)
F2	1,683,746	495,184 (29.41%)	24,876,188	17,447,088 (70.14%)
F3	1,525,532	598,882 (39.26%)	39,389,815	27,338,407 (69.40%)

for each according to the coordinates of the precursor RNA. The chromosome, start and end positions, sequence, sRNA length, hairpin length and minimum free energy for each novel miRNA were predicted.

Identification of differentially expressed miRNAs

In order to identified differentially expressed miRNAs between the back and belly skin in Rex rabbits, differentially expressed miRNAs with a $|\log_2$ (fold change)$|>1$ and *P*-value<0.05 were considered significant. Consequently, 211 miRNAs (90 upregulated miRNAs and 121 downregulated) were found to be significantly differentially expressed between the back and belly regions of the Rex rabbits (Table S4).

Prediction and analysis of miRNA target genes

After the differentially expressed miRNAs were identified, 7799 differentially expressed miRNA target genes were predicted by miRanda prediction software (Table S5). GO enrichment and KEGG pathway analysis were performed to determine the functions of the miRNA target genes (Table S6). Significance was set at a *P*-value <0.05 for the GO term analysis and target genes were found in three GO categories (biological processes, molecular functions, and cellular components). In biological process, the GO terms consisted of DNA–template, regulation of transcription, metabolic process, signal transduction, transmembrane transport and proteolysis, positive regulation of cell proliferation and positive regulation of target gene expression. In the molecular functions category, protein binding, metal ion binding, nucleotide binding, transcription factor activity, transferase activity, transferase activity and sequence–specific DNA binding had the highest enrichment of the differentially expressed miRNA target genes. In the cellular components category, membrane, cytoplasm, nucleus, integral component of membrane, extracellular exosome and plasma membrane contained a high proportion of these GO term categories (Figure 2). With the KEGG pathway analysis, the target genes of signalling pathways were enriched (*P*-value of genes is shown in Figure 3). Hair follicle and skin development related pathways were found, for example Wnt signalling pathway, Hedgehog signalling pathway, Osteoclast differentiation and MAPK pathway. The GO terms and KEGG pathways of target genes provide clues for understanding miRNA function and give further information on candidate miRNAs that play important roles in fur development on different parts of the body (Table S7).

Validation of differentially expressed miRNAs and predicted target genes by qRT-PCR

Nine differentially expressed miRNAs between the 2 groups were selected for validation. As shown in Figure 4, miR-34, miR-192, miR-215, miR-483, miR-6786-5p, miR-690, miR-1227 were downregulated, while miR-241, miR-876-3p were upregulated. The expression of the U6 small nuclear RNA was used for normalisation. Upregulated mRNA plays an important role in skin development correlating with the downregulation of miRNAs. As shown in Figure 5, five predicted target genes were chosen for the gene expression analysis by qRT-PCR. These include miR-1227 that targets Wnt16, miR-6786-5p that targets SFRP2, miR-192 that targets LUC7L, miR-34 that targets NFATC4 and miR-483 that targets Wnt10a. All 5 of these predicted target genes were found to be significantly upregulated in back group. These data suggest that miRNA profiling results were consistent with the overall changes in the differentially expressed miRNAs.

Network analysis between miRNAs and mRNA

To illustrate the relationship between miRNAs and their target genes involved in skin development, a network was constructed by analysing GO enrichment and signalling pathways of the differentially expressed miRNAs and their target genes. These miRNA and target genes were related to the development of hair follicles, skin and keratinocytes, hair follicle placode formation, the pathways such as Wnt signalling pathway or Hedgehog signalling pathway (Figure 6).

DISCUSSION

Increasingly, research has focused on the function and expression of miRNAs in a variety of species, and the expression of miRNAs is specific to different body parts in animals (Bashirullah *et al.,* 2003). In Chinchilla Rex rabbits,

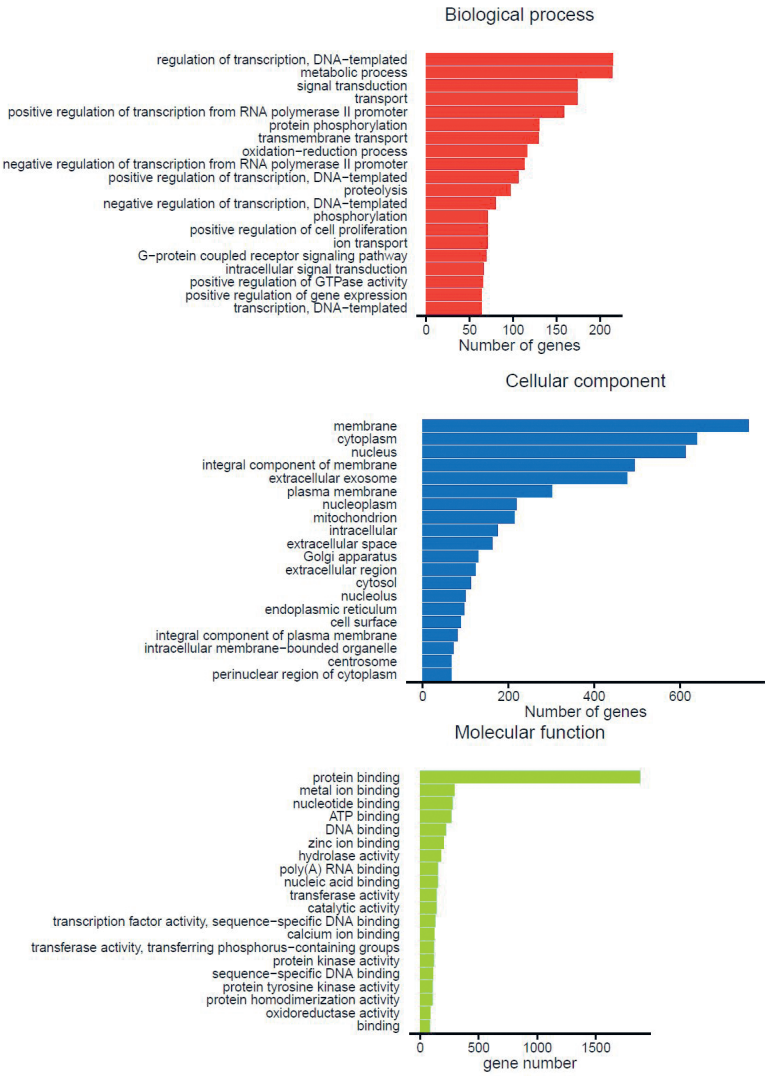

Figure 2: GO term categories for the most abundant miRNAs.

different traits exist between the back and belly skin, including different coat colour, length and diameter of wool (Tao, 2010), but there are very few studies on rabbits regarding the functional mechanism of miRNAs required for fur development in different body parts. However, several studies have shown the expression profiles of miRNA that play roles in skin development in different areas of an animal's body. A transcriptomic analysis of miRNAs in Cashmere goats identified 316 known miRNAs and 22 novel miRNAs, including miR-379, which that may have effects on skin, and HF and miR-127, which may play a key role in apoptosis and proliferation in skin follicle cells (Liu *et al.,* 2012). Several key miRNAs, which are related to morphogenesis of feather follicles and skin, were obtained by miRNA profiling in ducks (Zhang *et al.,* 2013). Many miRNAs were defined as epithelial-specific, including miRNA-205, miR-203, miRNA-31 and miRNA-34, and the interaction of endocrine factors and these miRNAs in the regulation of keratinocyte differentiation was analysed (Antonini *et al.,* 2010; Mardaryev *et al.,* 2010; D'Juan *et al.,* 2013; Jackson *et al.,* 2013). HF cycling is a complex biological process, involving 3 stages (anagen, catagen and telogen), and the temporal-spatial expression of miRNAs was evaluated, with miR-31 found to increase during anagen, but

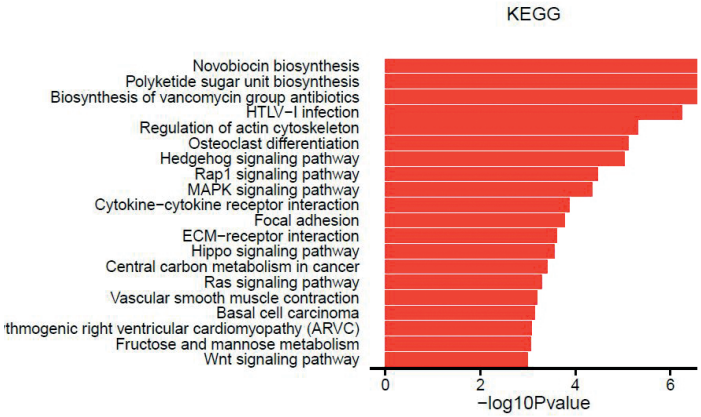

Figure 3: KEGG pathway analysis for the most abundant miRNAs.

decrease during catagen and telogen (Mardaryev *et al.,* 2010). During the development of HF and the epidermis, the formation of pigmentation is in progress and melanogenesis is regulated by many factors, in which miRNAs may serve as regulators between these factors and pigmentation. For example, skin colour related miRNAs have been found in brown and white skinned alpacas (Tian *et al.,* 2012). Using miRNA microarray experiments on melanocytes, miR-17-5p, miR-194, miR-22, miR-222 and miR-373 were found to be highly expressed in melanoma cell lines and melanocytes (Mueller *et al.,* 2009).

In this study, 211 miRNAs (90 upregulated and 121 downregulated) were identified, and may play important roles in the regulation of skin development between the backs and bellies of rabbits, including several miRNAs related to coat colour, hair length and HF development. We found miR-192 and miR-215 was highly expressed in the belly, meaning that miR-192/215 may be involved in the formation of coat colour and development of HF and skin. In previous studies, miR-192 was found to be expressed at a low level in melanoma cells (Caramuta *et al.,* 2010). Tumour protein p53, also known as p53, is a tumour suppressor that regulates the cell cycle in multicellular organisms (Vousden and Lane, 2007). By regulating p53, miR-192 has effects on cell proliferation and the cell cycle through

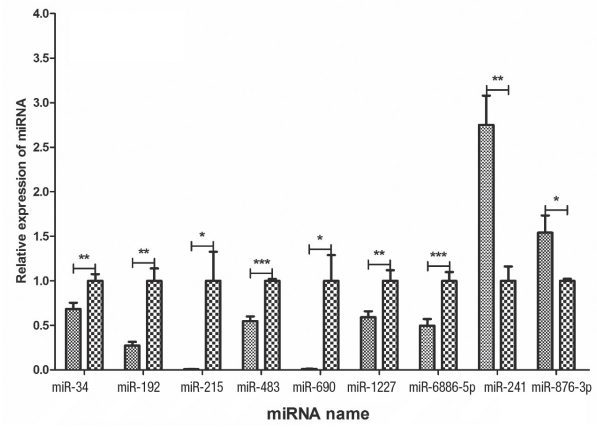

Figure 4: Validation of the differentially expressed miRNAs by qRT-PCR. The expression level of miRNAs in the back group was normalised to the belly group. Error bars represent the mean±standard deviation of triplicate experiment. **P*<0.05; ***P*<0.01; ****P*<0.001. Back group; Belly group.

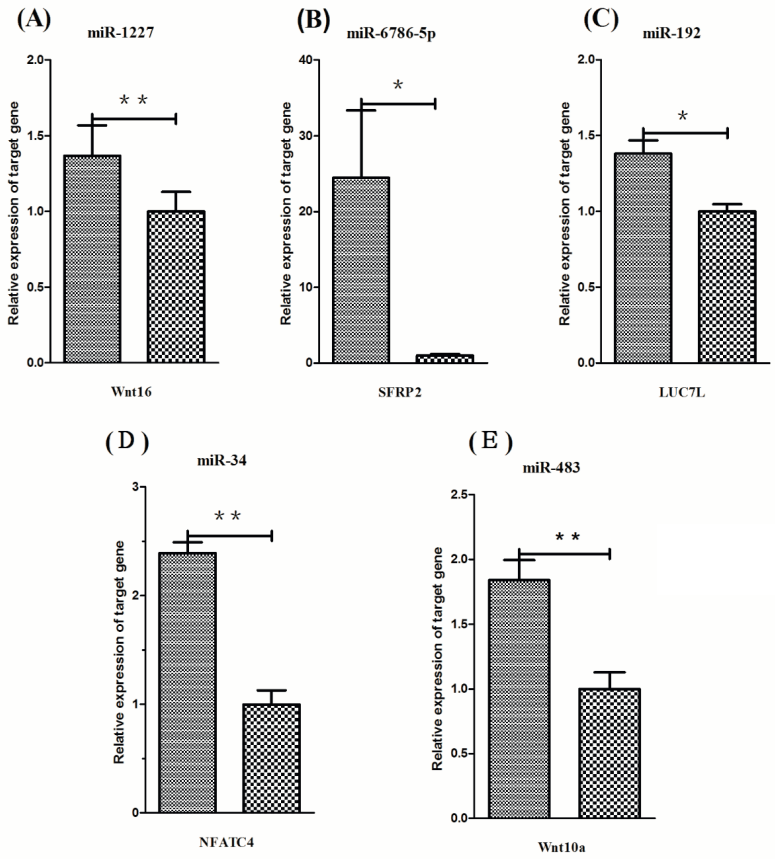

Figure 5: Validation of candidate target genes by qRT-PCR. Expression of predicted target genes of miR-1227 (A), miR-6786-5p (B), miR-192 (C), miR-34 (D) and miR-483 (E). The expression level of mRNA in the back group was normalised to the belly group. Error bars represent the mean±standard deviation of triplicate experiment. **P*<0.05; ***P*<0.01. Back group; Belly group.

the p53-miRNA circuit. In the p53 tumour suppressor network, miR-192 as a candidate factor affects cell activities, and it can promote cell cycle arrest, cell detachment and colony suppression (Braun *et al.,* 2008; Song *et al.,* 2008). Meanwhile, miR-215 is also regulated by p53, leading to cell cycle arrest (Georges *et al.,* 2009). Therefore, miRNAs play an important role in the regulation of cell cycle arrest in epithelial cells.

As a member of the p53 family, p63 an important factor in epidermal morphogenesis and is related to keratinocyte proliferation (Mills *et al.,* 1999; Yang *et al.,* 1999; DeYoung *et al.,* 2006; Senoo *et al.,* 2007). The disruption of p63 can depress the development of the epithelium, as seen in a recessive genetic model of p63 mice, which exhibit non-regenerative differentiation and the absence of squamous epithelia. This means p63 is a necessary factor to sustain epithelial development and morphogenesis (Yang *et al.,* 1999). Recent studies have shown that miR-34 maintains the process of proliferation, and activates cell cycle arrest, senescence and apoptosis by affecting the p53 function (Bommer *et al.,* 2007). Furthermore, miR-34 is expressed in keratinocytes of newborn mouse skin, the downregulation of miR-34 prevents cell cycle arrest when p63 is absent, and p63 represses the expression of miR-34 family members that regulate cell cycle progression in keratinocytes (Antonini *et al.,* 2010). In the belly group, miR-34 was expressed at a higher level than in the back group, suggesting that miR-34 counteracts the development of skin and HF.

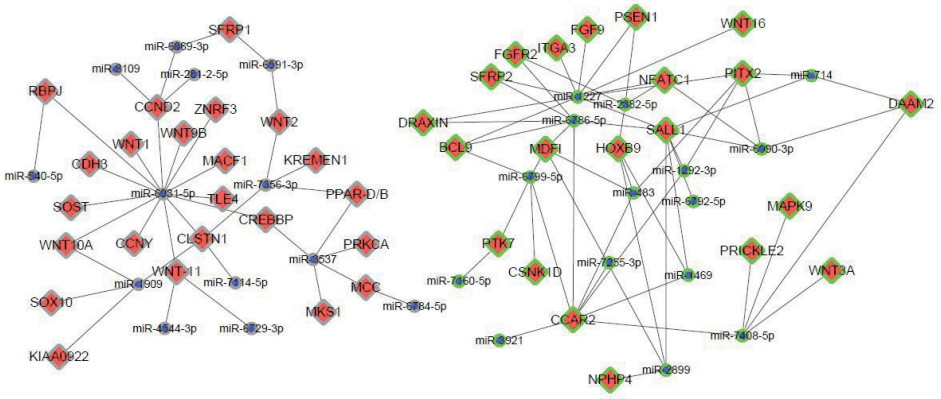

Figure 6: Network analysis between miRNAs and mRNA. A network showing the interaction of differentially expressed miRNAs regulating significant GO categories. Regulatory network of differentially expressed miRNAs and intersecting target genes associated with significantly enriched GO categories and signalling pathways. A: up-regulated miRNAs and their targets genes. B: down-regulated miRNAs and their targets genes. Note: Rectangles with rounded corners represent differentially expressed miRNAs; circles represent genes; lines represent regulatory relations between differentially expressed miRNAs and genes. Size of each node is related to the degree of relatedness.

Several miRNAs may regulate skin development in different cell populations; however, few studies have been performed on the interaction of genes and miRNA in skin development. Target genes that were regulated by differentially expressed miRNAs were identified by software prediction, and then the function of the target genes was analysed. The target genes play important roles in cell activities, such as cell cycle, cell proliferation, cell-cell signalling, cell development and differentiation, and an enrichment of genes involved in skin development, epithelial cell proliferation, hair follicle development and morphogenesis and melanogenesis was seen. Many target genes were related to keratinocyte differentiation, proliferation and migration, including CLIC4, VDR, Wnt5a, Wnt16, PPARD, VDR, CD109, FERMT1 and HB-EGF. The expression of CLIC4 is increased in differentiated keratinocytes in humans and mice, and CLIC4 also has effects on cell cycle arrest and epidermal homeostasis (Suh *et al.,* 2007). In keratinocyte studies, VDR is necessary for keratinocyte proliferation and differentiation and hair cycling, especially as a key factor for anagen initiation (Chen *et al.,* 2001; Oda *et al.,* 2007). Furthermore, Wnt family genes participate in many activities in skin development, tissue morphogenesis, epidermis differentiation, and HF development through Wnt signalling pathways (Andl *et al.,* 2002; Fuchs, 2007, Lim and Nusse, 2013). Wnt activation of β-catenin regulates the downstream genes of the pathway, with corresponding changes in many biological activities, including cell cycle and proliferation (van Amerongen *et al.,* 2012; Vadlakonda *et al.,* 2014). PPARD is one of these downstream genes in the Wnt signalling pathway and has an effect on keratinocyte differentiation and growth, as well as skin development (Rosenfield *et al.,* 2001).

Many studies have reported that miRNAs can regulate the Wnt signalling pathway by targeting the Wnt pathway related genes to control the development of skin and hair follicles. MiR-214 has inhibitory effects on skin epithelium differentiation, HF development and keratinocyte proliferation by targeting β-catenin. MiR-27a-3p decreased the expression of β-catenin by targeting Wnt3a, causing the inhibition of melanogenesis (Ahmed *et al.,* 2014; Andl and Botchkareva, 2015, Zhao *et al.,* 2015). Meanwhile, signalling pathways were enriched in the analysis of miRNA target genes, and we found that many skin development signalling pathways showed significantly different expression, including the Wnt signalling pathway, melanogenesis hedgehog signalling pathway and MAPK signalling. Many MAPK signalling factors regulated cell proliferation and differentiation, keratinocyte differentiation and epidermal morphogenesis (Eckert and Welter 1996; Whelan *et al.,* 2012). The Hedgehog signalling pathway plays a key role in different stages of hair follicle and skin development, and miRNAs can regulate these molecular mechanisms (Athar

et al., 2006; Katoh and Katoh 2008; Kim *et al.,* 2014). The interactions of target genes among these signalling pathways can help us understand the mechanisms of miRNA regulation.

CONCLUSION

In this study, we identified differentially expressed miRNAs involved in skin development between the back and belly skin of Rex rabbits. MiRNAs play crucial roles in skin cell population, hair follicle morphogenesis, melanogenesis and keratinocyte differentiation, and skin development has unique characteristics, like the specificity in different body parts, developmental stages and individuals. In order to reveal the regulation of miRNAs, the relationship between miRNAs and mRNA should be identified in further studies.

Acknowledgments: This study was supported by the Modern Agricultural Industrial System Special Funding (CARS-44-A-1), the Priority Academic Programme Development of Jiangsu Higher Education Institutions (2014-134) and the General Programme of Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (16KJB230001).

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