



Identification of Differentially Expressed Genes between White and Black Skin Tissues by RNA-Seq in the Tibetan Sheep (*Ovis aries*)

Zhen-Yang Wu¹, Li Li¹, Yu-Hua Fu², Sheng Wang³, Qing-Ming An¹, Xiao-Hui Tang⁴, Xiao-Yong Du^{3,5,*} and Fei Zhou^{6,*}

¹Tongren University, Tongren 554300, Guizhou, PR China

²School of Computer Science and Technology, Wuhan University of Technology, Wuhan, 430070, Hubei, PR China

³Key Laboratory of Swine Genetics and Breeding of Ministry of Agriculture and Key Laboratory of Agriculture Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science, Huazhong Agricultural University, Wuhan, 430070, Hubei, PR China

⁴Tibet Agricultural and Animal Husbandry University, Linzhi 860000, Tibet, PR China

⁵Hubei Key Laboratory of Agricultural Bioinformatics, College of Informatics, Huazhong Agricultural University, Wuhan, 430070, Hubei, PR China

⁶School of Civil Engineering and Architecture, Hubei University of Arts and Science, Xiangyang 441053, Hubei, PR China

ABSTRACT

Tibetan sheep is one of China's three coarse-wool sheep, the wool is mainly used for the production of Tibetan carpets and its coat color as one of important economic traits which can affect wool price directly. In this study, to investigate the functional roles of genes in Tibetan sheep skin with different coat colors, we sequenced genes from six skin samples using Solexa sequencing. The RNA-Seq analysis generated 63,283,784 and 63,644,062 clean reads in black and white skin, respectively. A total of 60 differentially expressed genes (DEGs) were identified, providing evidence that the different coat color skin changed considerably. Among these differentially expressed genes, there were 18 up-regulated genes and 42 down-regulated genes in black skin tissue. Further analysis showed that these genes involved a series of biological processes such as "pigment biosynthetic process" and "pigment metabolic process". KEGG pathways were analyzed for the differentially expressed genes and show that Melanogenesis signaling pathway may affect the process of coat color formation. Quantitative PCR confirmed differential expression of thirteen genes, TYR, TYRP1, TYRP2, MITF, β -catenin, Wnt3a and so on. These results will expand our understanding of the complex molecular mechanisms of coat color in Tibetan sheep and provide a foundation for future studies.

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Authors' Contribution

XYD and FZ conceived and designed the experiment. LL and XHT collected samples. SW and QMA performed some experiments. YHF helped in data analysis and preparation of some figures. ZYW analyzed the data, prepared the manuscript and provided funding.

Key words

Tibetan sheep, Coat color, RNA-sequencing, Differentially expressed genes, Q-PCR.

INTRODUCTION

Tibetan sheep are mainly distributed in the Qinghai-Tibet Plateau and adjacent to Gansu, Qinghai and other places, due to temperature, light, precipitation, altitude and other factors, Tibetan sheep formed many different ecological types (or subtypes). The wool as one of the important economic products, different quality wool has different uses and economic value. In addition to the quality and yield of wool, coat color is one of its important

economic traits.

The study of the genetic mechanism of sheep coat color began at the beginning of the 20th century, and a large number of studies have shown that the coat color of sheep is controlled by multiple alleles at multiple loci and reveals certain inheritance laws (Terrill, 1947; Singh and Singh, 1971; Vsevolodov *et al.*, 1987). Through hybridization experiments, a part of the gene loci controlling sheep coat color was revealed. So far, 11 gene loci have been used to control the color of sheep (Renieri *et al.*, 2008). This work constructs the basic framework for controlling the coat color genes, which has a guiding role in the control of the genetic and breeding work of the coat color traits in sheep production.

With the continuous development of molecular biology

* Corresponding authors: duxiaoyong@mail.hzau.edu.cn; zhoufei-0521@qq.com
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and biological technology, coat color research change gradually from the phenotypic genotype to the molecular level of the study, such as Candidate gene strategy, gene linkage analysis and Genome-wide association study. Candidate gene strategy plays an important role in the regulation of important economic traits in sheep, such as sheep growth traits (An *et al.*, 2017; Sahu *et al.*, 2017), reproductive traits (Barakat *et al.*, 2017; Hao *et al.*, 2018), disease resistance (Sayre *et al.*, 2012; Brown *et al.*, 2013; Periasamy *et al.*, 2014), milk production trait (Moili *et al.*, 2013) and hair follicle growth and development (Guo *et al.*, 2009). In recent years, the use of candidate gene method for the study of coat color is increasing (Davila *et al.*, 2014; Fontanesi *et al.*, 2014; Zhang *et al.*, 2017; Mahmoud *et al.*, 2017). Candidate gene method is widely studied with mammalian coat color-related candidate genes such as MC1R, ASIP, KIT, TYR, TYRP1 and so on.

A large of genetic variants associated with complex diseases have been identified by the Genome-wide association analysis (GWAS). GWAS not only contributes significantly to disease research, but also plays an important role in locating and screening important economic traits of animals. Such as fleece weight (Fateme *et al.*, 2017), sheep supernumerary nipple phenotype (Peng *et al.*, 2017), disease resistance (Atlija *et al.*, 2016; Čalić *et al.*, 2017), reproduction traits (Martinez-Royo *et al.*, 2017; Abdoli *et al.*, 2018) and sheep hair follicle growth (Goher *et al.*, 2010). This method was also used in animal coat color analyzed. Fan *et al.* (2014) analyzed the two Chinese Holstein dairy cows using bovine 50K SNP chip, suggesting that KIT, IGFBP7, PDGFRA, MITF, ING3 and WNT16 genes may be related to coat color. On the sheep, Kijas *et al.* (2013) obtained 49034 SNP loci by GWAS and found that most of the loci were associated with the MC1R gene. Similarly, the research of coat color is still very little, especially in the research of sheep coat color, few reports.

In recent years, the rapid development of high-throughput sequencing technology makes it possible to quickly explore potential genes that control traits. Sheep and goat skin, hair follicle tissue research is also more and more. Kang *et al.* (2013) used RNA-Seq technique to analyze the transcriptome of the skin tissue of 1 month and 48 months of age, respectively. It was found that there were 37 differentially expressed genes in these two different periods. Probably related pathways are Notch, VEGF, Wnt, MAPK and so on. Fan *et al.* (2013) performed a transcriptome sequencing of 6 neonat sheep (3 in white and black) and found that 2235 known differential expression genes were associated with coat color. But the coat color research is still inadequate. Yue *et al.* (2015) performed de novo transcriptome sequencing of skin and annotated 22,164 unigenes. No studies have been attempted to

identify mRNAs affecting coat color in the Tibetan sheep.

In this study, we sequenced mRNAs from black and white skin collected from 1-year old crossbred white and black coat color sheep. These results provide new information on mRNA expression profiles in the sheep and identify possible mRNA regulated pathways related to pigmentation in hair follicles.

MATERIALS AND METHODS

Experimental animals and sample collection

In this study, we collected the samples of Tibetan sheep in Bayi Town, Linzhi area, Tibet Autonomous Region. To eliminate the genetic background, samples were collected from three 1-year old crossbred white and black coat colored Tibetan sheep. In the living state, cut the sheep hair with the shears and make skin tissue completely naked. Cleaned and disinfected the naked skin surface with 75% alcohol. In the use of scalpels quickly cut 1cm × 1cm size of the skin tissue after slaughter. Divided skin into smaller tissue and stored quickly in the tissue preservation solution (TaKaRa). The preservation of the organization into the ice box for temporary storage, transported to the laboratory after 4°C refrigerator storage for 12h and then placed at -80°C permanent preservation.

RNA extraction and library construction

Removed the skin from the tissue preservation solution after thaw the frozen tissue in the ice box, cut the skin tissue with scissors into powder, add 1mL Trizol reagent, the use of tissue dispersion machine for homogenization. The supernatant was transferred to a new RNase Free 1.5 mL centrifuge tube after centrifuged at 12,000 rpm. Total RNA was isolated from skin of Tibetan sheeps according to the manufacturer's protocol.

Quality and quantity of RNA was examined using a NanoDrop 2000/2000C (Thermo Fisher Scientific Inc., Waltham, MA, USA) and integrity was detected using agarose gel electrophoresis. Six samples (three from white skin tissue and three from black skin tissue) from three Tibetan sheep each were sent to Genergy Biotechnology Co., Ltd. (Shanghai, China) for RNA library construction and sequencing. Sequencing was performed using an Illumina HiSeq 2000 Genome Analyzer (Illumina Inc., Santiago, CA, USA). Libraries were constructed using a TruSeq Small RNA Samples Preparation kit (Illumina Inc., Santiago, CA, USA).

Data preprocessing

Sequencing-received raw image data were transformed by base calling into sequence data (raw data). The quality of the original raw sequencing data

obtained by Solexa deep sequencing was assessed using FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The clean reads were obtained by trimming the low-quality reads and eliminating reads with contaminants. Prior to mapping reads to the reference database, we filtered all sequences as follows: (i) remove adaptor sequences and low-quality sequences (the percentage of low quality bases with a quality value ≤ 5 was $>50\%$ in a read); (ii) remove the N $>10\%$ in a read (N means that cannot determine the base information); (iii) remove the 5' adaptor contaminated reads; (iv) remove reads that don't have 3' adaptor sequences and inserts; (v) remove the 3' adaptor sequences and (vi) remove the polyA /T/G/C reads.

Mapping reads to the reference genome

In this study, Tophat 2 (Kim *et al.*, 2013) was applied to align the clean reads to the sheep genome (the sheep genomic DNA is from the Ensemble genome database, <http://www.ensembl.org/>) using the algorithm was a spliced mapping. The algorithm could segment the samples without full-length matching. In addition, Tophat 2 can align the whole sequence to the genomic exon. Matching allows two mismatches, each reads allow multi hits ≤ 1 . Two databases were employed for sequence analysis, the ensemble sheep (*Ovis aries*) reference gene and reference genomic DNA.

Normalized expression levels of genes and screening of DEseq

The results of the Tophat 2 alignment were quantitatively analyzed using cufflink (Trapnell *et al.*, 2013) and the FPKM values for each genes were calculated. The mapped read counts for each gene were normalized for RNA length and for the total read number in the lane according to reads per kilobase of exon model per million mapped reads (RPKM), which facilitates the comparison of transcript levels between samples (Mortazavi *et al.*, 2008). The Cuffdiff under Cufflinks will be used to normalize the resulting FPKM values for the analysis of the differences between the samples.

Gene ontology (GO), pathway enrichment analysis and validation of differentially expressed genes

GO functional enrichment analysis and KEGG pathway analyses were performed using DAVID software (<http://www.cytoscape.org/>). Differentially expressed genes were confirmed with qPCR (Chen *et al.*, 2005). Six samples (three white skin samples and four black skin samples) from four sheep were used in qPCR analysis. The primers are shown in Table I. One microgram of total RNA from each sample was reverse-transcribed into cDNA

using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). GAPDH was used as the internal control. qPCR was performed using standard protocols on the Roche LightCycler 480 Real-Time PCR Detection System (Hoffmann-La Roche Ltd, Basel, Switzerland). The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression levels (Livak *et al.*, 2001).

Table I.- The information of gene primer.

Gene Name	Primer sequence
OVIS-TYR-F	AGCCTGTGCCTCCTCCAAGA
OVIS-TYR-R	CAGTGGTGCCGTGGACAGAA
OVIS-TYRP1-F	GATGGCAGAGATGACCGAGAGG
OVIS-TYRP1-R	GCGGCAGGTTCCACAGTTGT
OVIS-TYRP2-F	GGGCAGCAAGGCAAGACGAT
OVIS-TYRP2-R	CAAGGTGACTCGGCGGTTGTAG
OVIS- β -catenin-F	TGAGGACCTACACTTACGAGAA
OVIS- β -catenin-R	ACAGCACCTTCAGCACTCT
OVIS-KIT-F	CAACCAAGGCGGCAGACAAGA
OVIS-KIT-R	TCAGCAAGTCCTCCAGGTCCAG
OVIS-EDNRB-F	CGTCCGCAACACCGCAAGTT
OVIS-EDNRB-R	CCAGCACGAACACTAGGCAGGA
OVIS-KITL-F	ACCCAGGCAGTTTACTCC
OVIS-KITL-R	AAGCCACTATCTCCAAGTCC
OVIS-MC1R-F	CATCGCCAAGAACC GCAACCT
OVIS-MC1R-R	AGCCTCCAGCAGCAGCATGA
OVIS-SOX10-F	CGGGAAGCCTCACATCGACT
OVIS-SOX10-R	TCCAACCTCGGCCACGTC
OVIS-WNT3a-F	TCGCAGCCTGACTTCCG
OVIS-WNT3a-R	CACCATCTCTGAGGCGCTGT
OVIS-MITF-F	ACAGACGGCACCATCACCTTCA
OVIS-MITF-R	TGCTCCTTCGGCTGCTGGTT
OVIS-LEF1-F	ATCCTCTGGTCCACACTCAAGG
OVIS-LEF1-R	ACATCATCAGTCACACGGTTCC
OVIS-PKA-F	CGCAACCTCCTACAAGTG
OVIS-PKA-R	TTCTCTCATAGTCGTC AA
OVIS-GAPDH-F	ACGGCACAGTCAAGGCAGAGA
OVIS-GAPDH-R	CGCTCCTGGAAGATGGTGATGG

RESULTS

Overview of sequencing data

To identify differentially expressed genes in the two types of skin (one is white and other is black), six mRNA libraries were constructed for Solexa sequencing. A total of 140,746,212 raw reads and 126,927,846 clean reads were obtained after eliminating the low quality reads, containing N sequences and adaptor sequences. The basic

information of sequencing data is also listed in Table II.

Table II.- The information of raw data filtering.

Sample Name	Total Reads	Clean Reads	Clean Ratio	Q20	Q30
1B	22,470,265	20,266,893	90.19%	98.18%	93.79%
2B	23,752,698	21,415,951	90.16%	98.05%	93.51%
3B	23,966,791	21,600,940	90.13%	98.10%	93.64%
1W	20,643,079	18,624,824	90.22%	97.96%	93.10%
2W	26,010,571	23,463,755	90.21%	97.97%	93.15%
3W	23,902,808	21,555,483	90.18%	97.97%	93.14%

Table III.- The most abundantly expressed gene in Tibetan sheep skin tissues (top 10).

Gene Names	Black skin	White skin	Sum
MT-ATP8	65192.9	130385.8	126843.1
KRTAP8-1	56621.7	113243.4	102791.2
KAP3.2	45360.2	90720.4	83371.3
COX1	30833.7	61667.46	60610.68
CST6	23010.4	46020.72	48337.67
KAP7	24826.9	49653.78	45179.5
MT-ATP6	19275	38550.06	37869.16
ZNFX1-AS1_2	18662.9	37325.78	37545.15
THYMB4X	18393.5	36787	36782.3
PRR9	18988.1	37976.12	36247.96

Gene expression levels analysis

Genetic quantification of the results of tophat alignment was performed using cufflink (VERSION: 2.1.1) and the FPKM values for each pair of genes were calculated. A total of 16,709 annotated genes were obtained. We list the top 10 genes with high expression levels in Table III. Found that these genes are ATP synthesis process, keratin and keratin associated protein, membrane-associated glycoprotein and other related.

Cuffdiff was used to analyze the normalized FPKM values between the samples. A total of 60 differentially expressed genes were obtained. Table IV lists only the top 10 up-regulated and the first 9 down-regulated genes.

Clustering of DEGs between the samples

To further analyze the differentially expressed genes, we do the heatmap of the differentially expressed genes (Fig. 1). It is convenient to see that there is no significant change between the two skin tissues (white skin and black skin). The clustering of the sample found that a black sheep skin sample and two other white skin tissue samples were clustered together to further indicate that there may be differences between individuals.

Table IV.- The information of differentially expressed gene between white skin and black skin in Tibetan sheep.

Gene names	White skin	Black skin	log ₂ (fold change)	p value
SNORD49	295.4	0	inf	0.00005
RETN	3.18	0	inf	0.00005
ZBP1	8.6	1.09	2.97	0.00005
MX2	41.91	5.36	2.97	0.00005
BST-2A	50.22	6.48	2.95	0.00005
HOXB6	10.16	1.53	2.74	0.00005
BST-2B	17.49	2.81	2.64	0.00005
ISG17	174.81	29.07	2.59	0.00005
RSAD2	25.75	4.44	2.53	0.00005
HOXB7	8.4	1.61	2.38	0.00005
PMEL	1.66	151.65	-6.51	0.00005
TYRP1	0.6	38.8	-6.02	0.00005
DCT	2.82	19.82	-2.81	0.00005
TYR	1.59	8.05	-2.34	0.00005
STMN2	2.37	11.52	-2.28	0.0001
VNN1	0.74	2.97	-2	0.00005
GPR143	3.4	13.08	-1.94	0.00005
OCA2	1.64	5.74	-1.81	0.00005
IL37	7.27	21.56	-1.57	0.00005

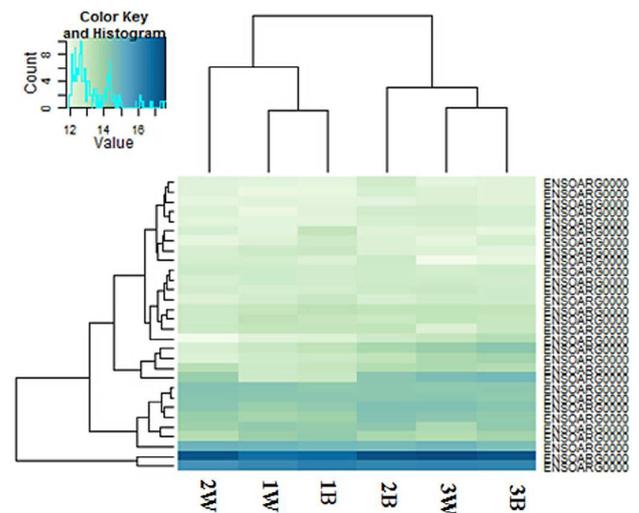


Fig. 1. The hierarchical clustering based on differentially expressed genes in Tibetan sheep skin between white and black coat color (top 30). 1W, 2W and 3W are the white skin samples from three Tibetan sheep; 1B, 2B and 3B are the black skin samples from three Tibetan sheep.

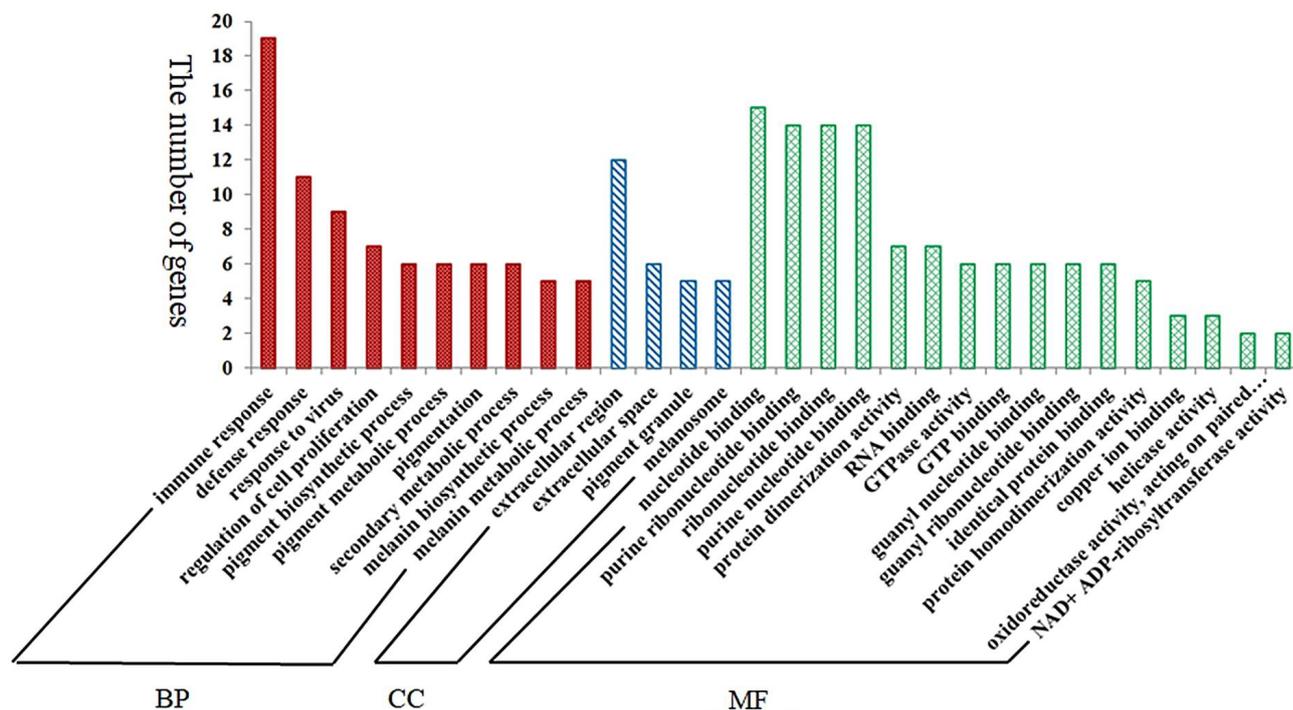


Fig. 2. GO analysis of differentially expressed genes in Tibetan sheep with white skin vs. black skin samples.

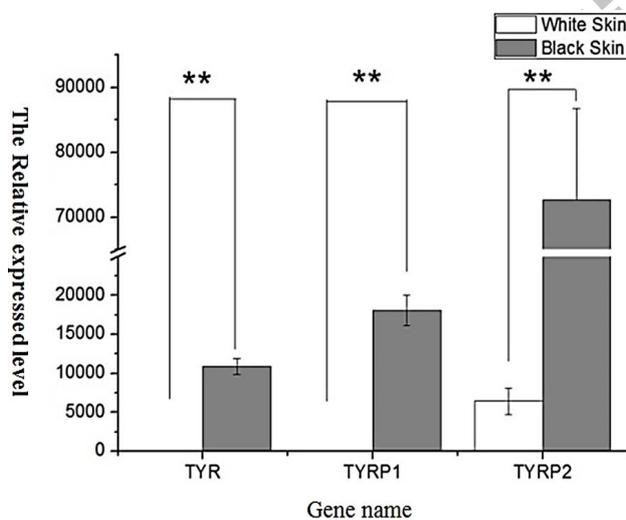


Fig. 3. Relative expression of three differentially expressed genes. * $P < 0.05$, ** $P < 0.01$.

Analysis of differential gene function

Genotyping of the differential genes obtained by cuffdiff was performed using the DAVID software for gene function analysis. We listed biological process (Fig. 2; Supplementary Table I), cellular component (Fig. 2; Supplementary Table II) and molecular function (Fig. 2; Supplementary Table III), respectively. Although

there are few differences in the expression of genes, but the results show that these genes in addition to “immune response”, “defense response” and “response to virus” and other biological processes related, there are many genes related to pigment deposition, such as “regulation of cell proliferation”, “pigment biosynthetic process”, “pigment metabolic process” and “pigment metabolic process”. In “cellular component” and “molecular function”, there are also genes associated with pigment deposition, such as “pigment granule” and “melanosome”.

The differentially expressed genes were analyzed by DAVID software to analyze the KEGG pathways of these differential genes. Only three KEGG pathways were obtained, namely “RIG-I-like receptor signaling pathway”, “Tyrosine metabolism” and “Melanogenesis”. The Melanogenesis pathway only covers three genes, TYR, TYRP1 and TYRP2.

Confirmation of differential gene expression by qPCR

We selected the most important three genes, TYR, TYRP1 and TYRP2 genes, to validate the expression profiles obtained by RNA-Seq. The qPCR results verified that these genes were differentially expressed at different color skins, consistent with the RNA-Seq findings (Fig. 3). Thus, RNA-Seq can provide reliable data for mRNA differential expression analysis from one side as with other similar findings.

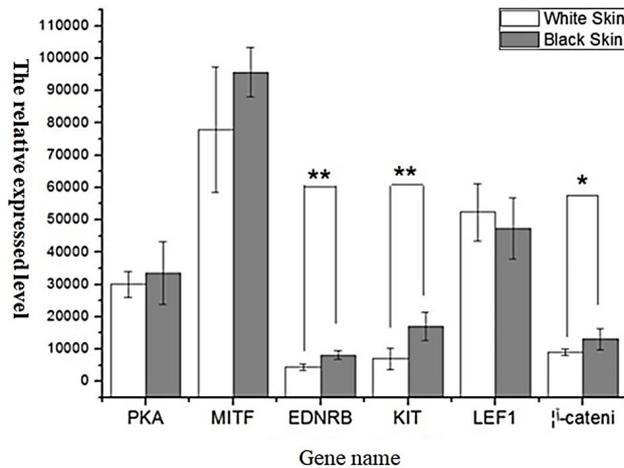


Fig. 4. Relative expression of six differentially expressed genes. * $P < 0.05$, ** $P < 0.01$.

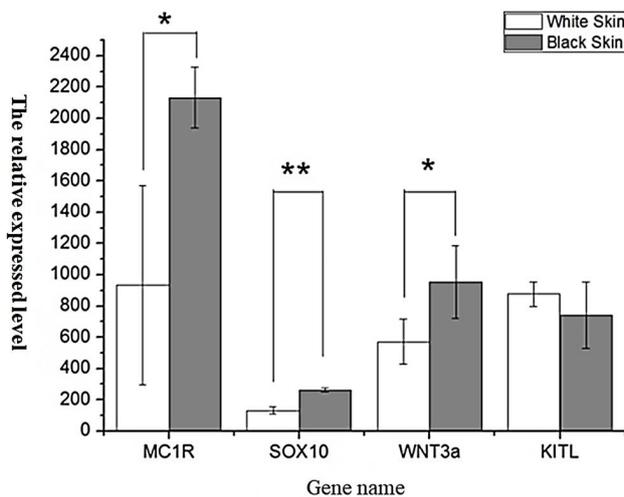


Fig. 5. Relative expression of four differentially expressed genes. * $P < 0.05$, ** $P < 0.01$.

We also selected candidate genes associated with melanocyte activity and melanin synthesis pathway for qPCR analysis. The genes included MC1R, KIT, KITL, MITF, WNT3a, SOX10, PKA, PAX3, EDNRB, β -catenin, and LEF1. Relatively high expression levels include PKA, MITF, EDNRB, Kit, LEF1 and β -catenin (Fig. 4). Among these genes, KIT and EDNRB showed extremely significant differences ($P < 0.01$), and β -catenin showed significant differences ($P < 0.05$), while other differences were not significant. KIT is considered to be an important factor in the migration of melanocytes' precursors, which can affect the activity of TYR in the later stage. MAPK and other signaling pathways can also be activated. However, in this study there was no difference in MITF gene, and

a large number of studies have shown that MITF is an important transcription factor in the process of melanin synthesis, which can promote the transcription of tyrosine gene family. Therefore, it was speculated that the upstream genes with inhibitory effect on TYR might influence its expression in other ways.

The genes with relatively low expression in skin tissues are MC1R, SOX10, WNT3a and KITL. SOX10 ($P < 0.01$) was the most significant difference, while WNT3a and MC1R ($P < 0.05$) were the most significant differences (Fig. 5). But based on differences in expression levels and multiples, these genes may not play a substantial role. Therefore, more genes need to be discovered to explain the formation mechanism of this complex trait.

DISCUSSION

In this study, we used the mRNA-Seq sequencing technique to sequence the skin tissues of three crossed black and white sheep, and obtain 70,189,754 and 70,556,458 reads in white and black skin, respectively. These samples were compared to the sheep genome and annotated, and then differentially expressed genes were analyzed by cuffdiff, and 60 differentially expressed genes were obtained. Among these genes, there were 18 up-regulated genes and 56 down-regulated genes in black skin tissues. Further analysis showed that these genes involved a series of biological processes such as "pigment biosynthetic process", "pigment metabolic process" and "pigment metabolic process". Fan *et al.* (2013) used a high-throughput sequencing technique to analyze the skin tissues of 6 Sunit ewes (3 black and 3 white), and received a total of 2,235 differentially expressed genes, of which 479 genes were in black Skin uptake expression, among these genes, there are 49 known control of the coat color genes. The expression of differentially expressed genes in this study was not high, but there was a similar result to Fan's work. We found that the distribution of data between the three groups may be different; it is possible to average the level of gene expression between the samples and impact the experimental results. Although the individual differences are large, but the results are still pointing to the sheep control the coat color by regulating the number of melanin. Melanin is produced by mature melanocytes, so there are two possible ways to reduce the production of melanin, one is through the regulation of melanoma stem cells into mature melanoma cells to achieve the reduction of the number of melanin, and other is the direct control of tyrosinase activity to control the amount of melanin (Yang *et al.*, 2016). The mature melanocytes of the skin and hair follicles come from the melanocytic stem cells of the hair follicle, and the melanocytic cells in the hair

follicles also change periodically as the hair follicles grow periodically, thus requiring the differentiation of melanocytes to supplement. The mature melanocytes of the skin and hair follicles come from the melanocytic stem cells of the hair follicle, and the melanocytic cells in the hair follicles also change periodically as the hair follicles grow periodically, thus requiring the differentiation of melanocytes to supplement. Melanocytes in the skin tissue are differentiated from the embryonic period of the neural crest cells, when the neural crest cell differentiation into melanocytes to the epidermal basal layer, the differentiation into mature melanoma cells, these melanocytes are generally not affected by hair follicle cycle the effect of growth changes has been stable in the basal layer of the skin (Kelsh *et al.*, 2009; Mayo *et al.*, 2014). But not all species of skin melanoma cells are so, so the epidermal melanoma cells and hair follicle melanoma cell regulation mechanism is a certain difference. We hypothesize that the reduction in the number of melanin in white hair follicles is likely to result in the lack of mature melanocytes in the hair follicles by controlling the differentiation of melanocyte stem cells. And skin tissue, it may be through the control of tyrosinase activity or the two controls caused by the combined effect. So it is necessary to carry out more in-depth study.

The expression levels of TYR, TYRP1 and TYRP2 were significant in the different skin tissues, and the expression level of TYR, TYRP1 and TYRP2 was very high in black skin tissue. The expression of TYR gene and TYRP1 gene in black skin was 46 times and 450 times higher than that of white skin, while that of TYRP2 was only 11 times. These three genes are melanoma cell-specific protein family members, respectively, catalyze the different stages of melanin biosynthesis. A large number of studies have confirmed that mutations in TYR can lead to whitening of many species such as sheep, mice, rabbit and so on (Song *et al.*, 2017; Xing *et al.*, 2015; Ming *et al.*, 2016). The TYRP1 gene is not well known at present and is located in the brown locus of mice and is also known as the Brown gene. Through the study of the function of TYRP1 gene, it was proved that the protein had DHICA oxidase activity, which played an important role in the downstream pathway of melanin synthesis. In addition, studies have shown that TYRP1 in addition to control the synthesis of pigment process, but also on the stability of tyrosinase and to maintain the ultrastructure of melanoma cells and melanoma cell proliferation and differentiation plays an important role (Hreckova *et al.*, 2017; Li and Li, 2015). The TYRP2 function has been identified as enabling the rapid incorporation of carboxylic acid precursors DHICA into biosynthetic melanin, which is important for reducing the cytotoxicity of these intermediates (Jiang *et al.*, 2016;

Andrzej *et al.*, 2016; Nie *et al.*, 2016). This study shows that these three genes are significantly different, especially the expression of TYR and TYRP1 genes. It is speculated that the difference between black and white coat color, whether in the melanin synthesis process, or in the process of proliferation and differentiation of melanoma cells are subject to varying degrees of block.

In black and white skin tissues of Tibetan sheep, β -catenin and Wnt3a were highly expressed in black skin, in which β -catenin in black skin was significantly higher than that in white skin ($P < 0.01$), while Wnt3a in black skin was significantly higher than that in white skin ($P < 0.05$), suggesting that the formation of different hair colors of Tibetan sheep may be related to the Wnt/ β -catenin signaling pathway. The study of Yu *et al.* (2010) in brown and white alpacas also showed that both mRNA expression and protein expression of β -catenin were significantly higher in brown skin than in white skin. Song *et al.* (2012) also used qPCR and Western blot analysis in brown and white alpaca skin and found significant differences in Wnt3a in skin tissues of different colors. Wnt3a is the ligand of the classical WNT signaling pathway. Both Wnt3a and the activated WNT/ β -catenin signaling pathway protein are expressed in the hair follicle melanocytes during the growth period, so they play an important role in the proliferation, differentiation and pigmentation of the hair follicle melanocytes. Studies in mouse melanin stem cells showed that Wnt3a activated the Wnt/ β -catenin signaling pathway, thus promoting the differentiation of McSCs in this process, and Wnt3a induced hair follicle regeneration (Guo *et al.*, 2016). A large number of studies have confirmed the important role of the Wnt/ β -catenin signaling pathway in the differentiation, proliferation and melanin production of melanocytes (Zhao *et al.*, 2014; Chung-Hsing *et al.*, 2014; Guo *et al.*, 2012). Among these genes, β -catenin is an important adhesion molecule in epithelial tissue and a widely distributed multifunctional protein involved in various developmental processes including neural crest. β -catenin also regulates the expression of MITF and can work with it to regulate the transcription of downstream genes (Cheong-Yong *et al.*, 2015). MITF gene was first discovered in mice in 1942, located on chromosome 6 and able to bind to relevant downstream targets to regulate the expression of downstream proteins, thus affecting the proliferation, survival and pigmentation of melanocytes (Liu *et al.*, 2017). However, the results showed that there was no significant difference in the expression of MITF gene in the skin tissues of Tibetan sheep with different colors ($P > 0.05$), indicating that MITF gene might not be involved in the hair color regulation of Tibetan sheep. Han *et al.* (2013) came to a similar conclusion, pointing out that the mRNA expression level of MITF gene was

significantly higher in the black skin tissues around the orbit of Tibetan sheep than in the brown-black skin tissues of the neck and the white skin tissues of the body side, while the mRNA expression level of MITF gene in the brown-black skin tissues of the neck and the white skin tissues of the body side was not significantly different. MITF promoter region contains binding regions of transcription regulatory factors such as SOX10 and LEF1, so it is also regulated by a variety of signal molecules. In the experiment, the expression of LEF1 gene in different hair colors was also not significantly different, while SOX10 gene was differentially expressed in skin tissues of different hair colors, but the expression level was very low. Therefore, it can be speculated that the hair color regulation of Tibetan sheep may be related to Wnt/ β -catenin signaling pathway, but not to MITF gene and its related transcription factors. MC1R can activate intracellular adenylate cyclase system, and then activate the tyrosine kinase, tyrosine (TYR) involved in the synthesis of melanin. Zhang *et al.* (2019) transfected MC1R plasmid of alpaca into sheep melanocytes, and not only found that mRNA and protein expression levels of relevant downstream genes such as TYR and TYRP1 in sheep melanocytes were significantly up-regulated, but melanin synthesis was also significantly increased. Tang *et al.* (2018) also found that MC1R gene expression in dark skin tissues was higher than that in white skin tissues in different color yaks. The expression of MC1R gene in black skin tissues of Tibetan sheep is significantly higher than that in white skin, indicating that MC1R gene can increase the content of melanin by up-regulating downstream genes such as TYR in the process of melanin synthesis, thus playing a regulatory role in hair color.

The formation of hair color is not only related to the production of melanin, but also to the migration, distribution and proliferation of melanocytes. The KIT gene encodes mast/stem cell growth factor receptors, which together with their ligand (KITL) play a critical role in the survival and maintenance of hematopoietic stem cells and mast cells. It also affects the proliferation, differentiation and migration of melanocytes, so it plays a crucial role in the formation of melanin. Among them, abnormal expression of KIT gene can directly affect the number of melanocytes, which is also one of the reasons for the formation of white fur (Anello *et al.*, 2019). The expression level of KIT gene in black skin of Tibetan sheep was significantly higher than that in white skin, while the expression level of KITL was not significantly different between the two skin tissues. The results showed that KIT gene plays an important role in the proliferation of melanocytes in Tibetan sheep skin. In addition to KIT gene, EDNRB gene was also highly expressed in the black

skin tissue of Tibetan sheep and was highly expressed in the white skin. This is consistent with the research of Geng *et al.* (2010). The translation products of EDNRB gene and functional signals of EDN3 are necessary for the development of pigment cells derived from neural crest, which play a role in signal transmission during the development of neural crest derived melanocytes and are important candidate genes related to hair color (Liu *et al.*, 2018).

CONCLUSION

In conclusion, the development of melanocytes is a complex event involving numerous genes and pathways that interact and show cross-talk. These studies will provide new insights into the regulation of coat color formation for understanding the regulatory mechanism at the post-transcription level.

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Supplementary material

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Supplementary Material

Identification of Differentially Expressed Genes between White and Black Skin Tissues by RNA-Seq in the Tibetan Sheep (*Ovis aries*)

Zhen-Yang Wu¹, Li Li¹, Yu-Hua Fu², Sheng Wang³, Qing-Ming An¹, Xiao-Hui Tang⁴, Xiao-Yong Du^{3,5,*} and Fei Zhou^{6,*}

¹Tongren University, Tongren 554300, Guizhou, PR China

²School of Computer Science and Technology, Wuhan University of Technology, Wuhan, 430070, Hubei, PR China

³Key Laboratory of Swine Genetics and Breeding of Ministry of Agriculture and Key Laboratory of Agriculture Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science, Huazhong Agricultural University, Wuhan, 430070, Hubei, PR China

⁴Tibet Agricultural and Animal Husbandry University, Linzhi 860000, Tibet, PR China

⁵Hubei Key Laboratory of Agricultural Bioinformatics, College of Informatics, Huazhong Agricultural University, Wuhan, 430070, Hubei, PR China

⁶School of Civil Engineering and Architecture, Hubei University of Arts and Science, Xiangyang 441053, Hubei, PR China

Supplementary Table I.- GO analysis of differentially expressed genes in Tibetan sheep with white skin vs. black skin samples(biological process).

GO accession	GO terms	Gene count	Percentage (%)	p-Value
GO:0006955	Immune response	19	27.94	1.07E-10
GO:0006952	Defense response	11	16.18	1.61E-04
GO:0009615	Response to virus	9	13.24	1.22E-08
GO:0042127	Regulation of cell proliferation	7	10.29	0.099
GO:0046148	Pigment biosynthetic process	6	8.82	4.78E-07
GO:0042440	Pigment metabolic process	6	8.82	9.96E-07
GO:0043473	Pigmentation	6	8.82	3.91E-06
GO:0019748	Secondary metabolic process	6	8.82	1.65E-05
GO:0042438	Melanin biosynthetic process	5	7.35	1.69E-08
GO:0006582	Melanin metabolic process	5	7.35	3.03E-08

Supplementary Table II.- GO analysis of differentially expressed genes in Tibetan sheep with white skin vs. black skin samples (cellular component).

GO accession	GO terms	Gene count	Percentage (%)	p-Value
GO:0005576	Extracellular region	12	17.65	0.074
GO:0005615	Extracellular space	6	8.82	0.085
GO:0048770	Pigment granule	5	7.35	2.41E-04
GO:0042470	Melanosome	5	7.35	2.41E-04

* Corresponding authors: duxiaoyong@mail.hzau.edu.cn;

zhoufei-0521@qq.com

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Supplementary Table III.- GO analysis of differentially expressed genes in Tibetan sheep with white skin vs. black skin samples(molecular function).

GO accession	GO terms	Gene count	Percentage (%)
GO:0000166	Nucleotide binding	15	22.06
GO:0032555	Purine ribonucleotide binding	14	20.59
GO:0032553	Ribonucleotide binding	14	20.59
GO:0017076	Purine nucleotide binding	14	20.59
GO:0046983	Protein dimerization activity	7	10.29
GO:0003723	RNA binding	7	10.29
GO:0003924	GTPase activity	6	8.82
GO:0005525	GTP binding	6	8.82
GO:0019001	Guanyl nucleotide binding	6	8.82
GO:0032561	Guanyl ribonucleotide binding	6	8.82
GO:0042802	Identical protein binding	6	8.82
GO:0042803	Protein homodimerization activity	5	7.35
GO:0005507	Copper ion binding	3	4.41
GO:0004386	Helicase activity	3	4.41
GO:0016716	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen	2	2.94
GO:0003950	NAD+ ADP-ribosyltransferase activity	2	2.94