

## Differential expression of genes and proteins associated with wool follicle cycling

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**Abstract** Sheep are valuable resources for the wool industry. Wool growth of Aohan fine wool sheep has cycled during different seasons in 1 year. Therefore, identifying genes that control wool growth cycling might lead to ways for improving the quality and yield of fine wool. In this study, we employed Agilent sheep gene expression microarray and proteomic technology to compare the gene expression patterns of the body side skins at August and December time points in Aohan fine wool sheep (a Chinese indigenous breed). Microarray study revealed that 2,223 transcripts were differentially expressed, including 1,162 up-regulated and 1,061 down-regulated transcripts, comparing body side skin at the August

time point to the December one (A/D) in Aohan fine wool sheep. Then seven differentially expressed genes were selected to validate the reliability of the gene chip data. The majority of the genes possibly related to follicle development and wool growth could be assigned into the categories including regulation of receptor binding, extracellular region, protein binding and extracellular space. Proteomic study revealed that 84 protein spots showed significant differences in expression levels. Of the 84, 63 protein spots were upregulated and 21 were downregulated in A/D. Finally, 55 protein points were determined through MALDI-TOF/MS analyses. Furthermore, the regulation mechanism of hair follicle might resemble that of fetation.

**Keywords** Microarray · Proteomic technology · Wool growth cycling · Differential expression

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Nan Liu and Hegang Li contributed equally to this paper.

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## Introduction

DNA microarray analysis has been proved to be an effective method in investigating the characteristics of hair follicle stem cells in mice [1–4]. A serial of skin-expressed microRNAs with possible roles in goat and sheep wool growth has also been described [5]. Yu et al. [6, 7] testified the effectiveness of cDNA chip for expression profiling of wool growth cycling in whole skin. Transcriptomic studies on sheep and goats have been reported, related to different traits, such as wool follicle development [8], pigmentation traits of skin and wool [9], natural fleece rot resistance [10], mammary development and milk quality [11–13], and resistance to parasites [14, 15]. Comparative transcriptomic analysis of the primary and secondary follicles of a cashmere goat reveal 51 genes that are differentially expressed between the two types of hair follicles [16].

Fine wool sheep provides high quality wool for the industry. Identifying genes that regulate wool growth cycling helps to find a way to improve the quality and yield of wool products in breeding programs. Aohan fine wool sheep, bred in Inner Mongolia, is an excellent breed, supplying both fine wool and meat. The advantages of this breed is high-quality wool, high resistance to disease and strong ability to adapt. Ultimately, Aohan fine wool sheep is a preferable breed for fine wool production in China. Wang et al. [17] demonstrated that climate factors displayed appreciable impact on wool growth of Aohan fine wool sheep. The peak of growth rate is in summer and the low in winter [17]. That is to say, the wool growth has cycled during different seasons in a year. The expression profiling of immune genes and type I inner root sheath (IRS) keratin genes in the whole skin of Aohan fine wool sheep has been described previously by our lab [18, 19].

Hair follicle is a regenerating system. The follicle demonstrates the unusual ability to completely regenerate itself. The basis for this regeneration rests in the unique follicular epithelial and mesenchymal components and their interactions [20]. Yu et al. studied the wool follicle cycling through adjusting the artificial illumination time [6, 7]. That is only part of the explanation, however. After all, natural lighting could provide a preferable answer.

As far as we know, no microarray or proteomic study at a genome-wide level has been conducted on genes that were possibly responsible for controlling wool growth cycling of adult sheep at natural conditions to date. The purpose of this study was to investigate and compare the gene expression level of body side skin at different time points through gene chip and proteomic technology, and to search and select the candidate genes and proteins responsible for wool growth cycling control of Aohan fine wool sheep.

## Materials and methods

### Animals and sample preparation

All animals were handled according to the animal processing protocols approved by national and local animal welfare agencies, and all animal experiments was authorized by the Shandong Province Biological Studies Animal Care and Use Committee.

Sampling methods were described previously [18, 19, a paper submitted to BMC Genetics]. One ram and two ewes were used in the cDNA microarray study. These animals were half sibs (sharing the same father). In August and December time points of 2010, whole skin from body side area (wool bearing) were picked up from the three animals under local anaesthesia for cDNA microarray and proteomic experiments. All samples were put into liquid nitrogen immediately after collection. The area of each skin sample was approximately one square centimetre. There were 15, 208 probes in Agilent Sheep Gene Expression Microarray (Santa Clara, CA, USA).

### RNA extraction and microarray hybridization

Total RNA was extracted from the skin samples using TRIzol reagent (Invitrogen) following the manufacturer's protocols. RNA concentration was assessed with a NanoDrop spectrophotometer (NanoDrop Technologies) and RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent). The RNA samples were then incubated with the Agilent Sheep Gene Expression Microarray (Santa Clara, CA, USA). Each RNA sample was hybridized to one microarray slide (one-to-one). 1 µg of total RNA from each sample was doubled and transcribed into fluorescent cRNA using the manufacturer's Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The marked cRNAs were added onto the Whole Genome Oligo Array (4×44K, Agilent Technologies). All the microarray experiments were performed by Kangchen Biotechnology Limited Company (Shanghai, China).

### Microarrays data analysis

After hybridization and washing, the microarray slides were scanned with the Agilent Scanner G2505B. The resulting text files derived from Agilent Feature Extraction Software (version 10.5.1.1) were introduced into the Agilent GeneSpring GX software (version 11.0) for subsequent calculation. Gene expression levels were standardized relative to the level of GAPDH. Differentially expressed genes were found out through fold-change screening. The fold-change of 2.0 were set up as the threshold. The statistical analysis was performed with Student's *t* test and then the *P*-values were corrected for multiple testing. The false discovery rate was set as approximately 20 %, in order to get more

differentially expressed genes for further analysis. The functional annotation of differentially expressed genes was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) gene annotation tool (<http://david.abcc.ncifcrf.gov/>) [21].

#### qPCR

The total RNA samples prepared for microarray detection were also used for qPCR experiments. Reverse transcriptions were performed using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocols. The primers were designed using the Primer Premier 5 software. The primer sequences, melting temperatures and product sizes are shown in Table 1. GAPDH was used as the house keeping gene.

#### Tissue protein extraction

Lysis buffer was prepared using ddH<sub>2</sub>O as dissolvent as follows: 42 % urea, 15.2 % thiourea, 4 % CHAPS, 1 % DTT. The buffer was usually separated into 1 ml volume and stored at –20 °C. The skin samples were cut into small pieces by scissors, and left at 4 °C for 1 h, vortexed it every 15 min. Sampled tissues were homogenized in lysis buffer (containing 1 % cocktail and 2 % IPG-buffer, added right before use) at the ratio of 1:7 (w/v). Then, the tissue homogenate was centrifuge at 40,000 g for 30 min. Supernatants were saved and stored at –70 °C. The protein concentration in each sample was determined by the bicinchoninic acid (BCA) method using PBS as the standard.

#### Two-dimensional electrophoresis (2-DE)

The electrophoresis programme is as follows:

Transfer: voltage 300 V, current 50 mA, time 1 h.

Separation: voltage 300 V, current 200–250 mA, time 4–5 h.

Fixative preparation: 40 % ethanol and 10 % acetic acid.

Fixation: take out the rubber strip and put it into fixative for 1 h.

#### Image analysis

Gels were silver stained, scanned and analyzed using ImageMaster TM 2D platinum software (Version 5.0, GE Healthcare, San Francisco, CA, USA). The expression level was determined by the relative volume of each spot in the gel and expressed as %Vol (%Vol = [spot volume/Σvolumes of all spots resolved in the gel]). The means and standard deviations of both sample groups were calculated. Statistical significance with Student's *t* tests using ImageMaster TM 2D platinum software. The *P* values obtained with the *t* test were corrected for multiple testing. The false discovery rate was set as approximately 20 %, in order to get more differentially expressed proteins for further analysis.

#### Protein identification

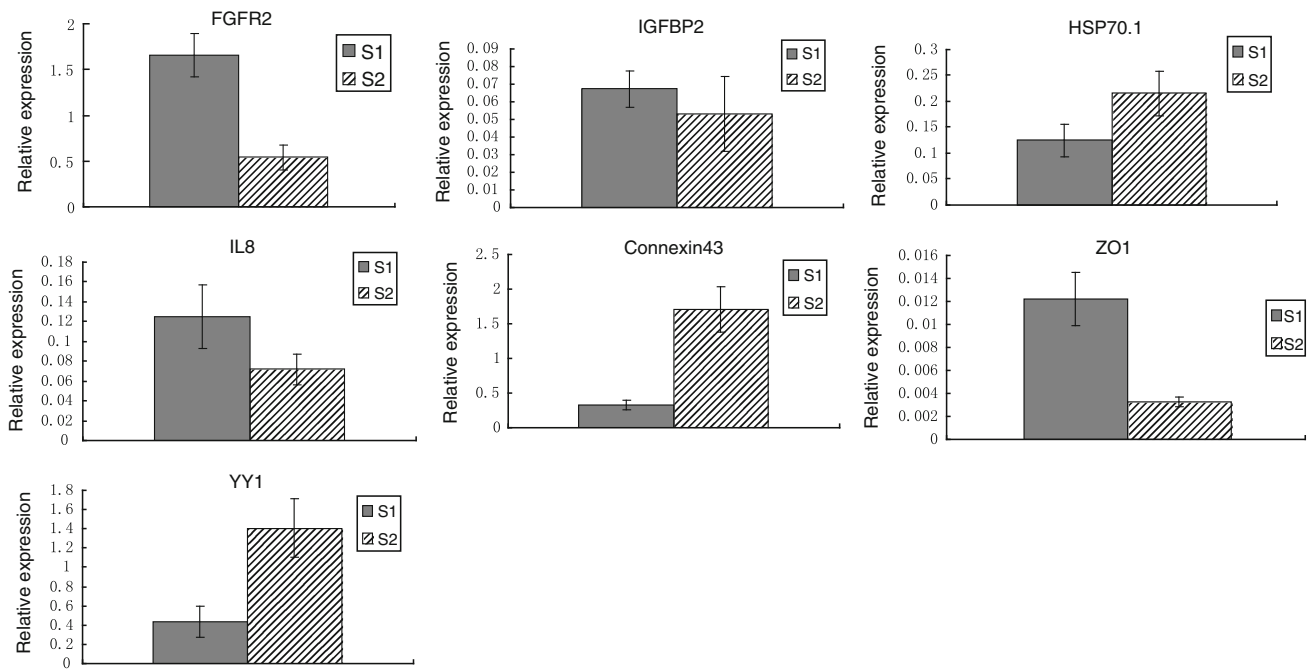
Protein spots were excised, dehydrated in acetonitrile, and dried at room temperature. Proteins were reduced and alkylated. Gel pieces were denatured, alkylated, trypsin

**Table 1** Primers used for Q-PCR validation

Gene	Primer sequence (5'-3')	Tm (°C) <sup>a</sup>	Target size (bp)
GAPDH <sup>b</sup>	Forward: ATGCCTCCTGCACCACCA	60	76
	Reverse: AGTCCCTCCACGATGCCAA		
FGFR2	Forward: ATACGTGCTTGCCGGGTAAT	60	210
	Reverse: TGAAGTCCGGCTTCTTGGTC		
IGFBP2	Forward: CGAGCAGGTTGCAGACAATG	60	379
	Reverse: GAGGTTGTACAGGCCATGCT		
Connexin 43	Forward: GTCGTGTCGTTGGTGTCTCT	60	291
	Reverse: CACTCAGCGTGTCCAGTTCT		
Zo1	Forward: AGATAGCCCTGCAGCCAAAG	60	117
	Reverse: GGGAGGTCAAGCAGGAAGAG		
HSP70.1	Forward: CCACGAAGCAGACGCAGAT	60	104
	Reverse: CAGGTTGTTGTCCCGAGTCAT		
IL8	Forward: AGAGCTGAGAAGCAAGATCCA	60	150
	Reverse: AACCCCTACACCAGACCCACA		
YY1	Forward: GCATTGACCTCTCAGACCCC	60	111
	Reverse: TGCAGCCTTTATGAGGGCAA		

<sup>a</sup> The annealing temperature represents the optimal temperature during quantitative PCR

<sup>b</sup> RNA levels of GAPDH was assayed for normalization during quantitative PCR



**Fig. 1** Q-PCR validation of the microarray data. *P* values (*T*-test) of the Q-PCR data are 0.021 (*FGFR2*), 0.157 (*IGFBP2*), 0.010 (*Connexin 43*), 0.023 (*ZO1*), 0.035 (*HSP70.1*), 0.042 (*IL8*) and

0.026 (*YY1*), respectively. S1/S2 represent body side skin at the August and December time points, respectively

digested and analyzed by the Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) under the control of FlexControl TM 2.4 software (Bruker Daltonics GmbH). Acquired peptide mass fingerprint (PMF) were analysed with the program Flex-Analysis™ 3.0 (Bruker Daltonics, Bremen, Germany). The peak detection algorithm was: SNAP (Sort Neaten Assign and Place); S/N threshold: 1.5; Quality Factor Threshold: 50. The tryptic auto-digestion ion picks (trypsin [108–115] 842.51 Da, trypsin [58–77] 2211.10 Da) were used as internal standards to validate the external calibration procedure. Matrix and/or auto-proteolytic trypsin fragments and known contaminant ions, were excluded. The derived peptide mass lists were used to aligning the Matrixscience database (<http://www.matrixscience.com>). The following search parameter criteria were used: mass tolerance 100 ppm, miss cleavage  $\leq 1$ , modification comprises carbamidomethyl and methionine oxidation. Matched peptides number between experimental PMF and theoretical PMF  $\geq 5$  [22].

## Results

### Summary of differentially expressed genes in microarray analysis

Two thousand two hundred and twenty-three annotated transcripts were differentially expressed between the two

time points, including 1,162 up-regulated and 1,061 down-regulated transcripts, as shown in Table S1 (Supplementary material). 24 genes were up-regulated more than 10 folds, while 16 genes downregulated more than 10 folds, in August time point versus December time point (A/D).

Furthermore, as shown in Table S2 (Supplementary material), many gene families that regulate different aspects of hair follicle growth and cycling show differential expression in A/D, such as growth factors, immune genes, enzymes, pregnancy-associated glycoprotein, and so on.

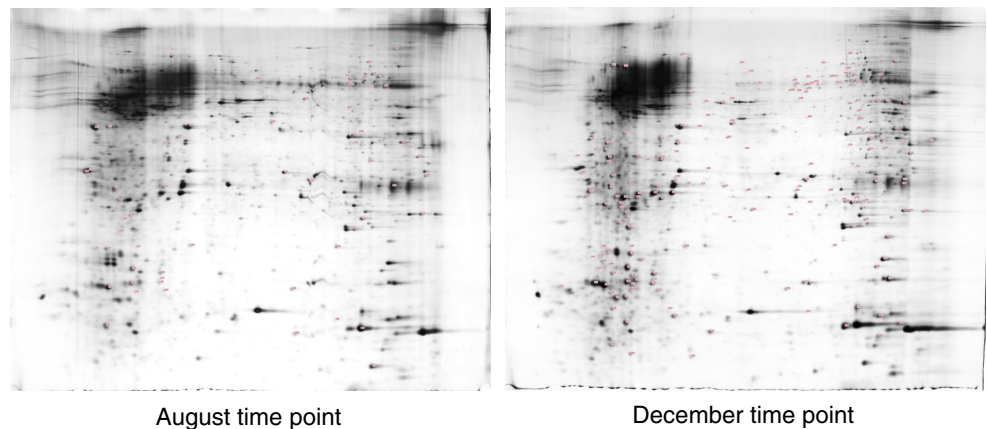
### Biological process gene ontology (GO) analysis

Based on the DAVID, scores of genes classified into 9 categories (Table S3 in Supplementary material). The majority of the genes possibly related to follicle development and wool growth could be assigned into the categories including regulation of receptor binding, extracellular region, protein binding and extracellular space.

### Selective verification for microarray data by qPCR

To confirm the data on the transcriptional level, we picked up 7 DE genes, including *FGFR2*, *IGFBP2*, *Connexin 43*, *ZO1*, *HSP70.1*, *IL8* and *YY1* to comparatively detect their expression patterns by real time PCR. As illustrated in Fig. 1, the qPCR results for the selected 6 genes were consistent with the microarray data except *IGFBP2*, which verified the credibility of the microarray data.

**Fig. 2** Representative image of 2-DE silver stained polyacrylamide gel



### Identification of DE protein spots on 2-DE gels

To detect differential protein expression between the two groups, triplicate 2D maps of protein samples for each group were created. Figure 2 shows two representative 2-DE gel images of the protein expression patterns of the two groups. 84 protein spots showed significant differences in expression levels between the two groups. Of the 84, 63 protein spots were upregulated and 21 were downregulated in A/D. Some of them could not be identified causing by incomplete polypeptide fragments or low abundance (beyond the detection limit). Finally, 55 protein points were determined through MALDI-TOF/MS analyses. These DE proteins is displayed in Table S4 (Supplementary material).

### Discussion

#### Significance of differentially expressed genes at the transcriptional level

In this study, we investigated the differentially expressed genes and proteins possibly regulating wool growth and cycling using cDNA microarray and proteomic technologies. Gene chip analysis identified hundreds of differentially expressed genes showing more than twofold difference between the two time points: August and December. While upregulated transcripts (1,162) in A/D was a little more than that of the downregulated ones (1,061). The qPCR experiments verified the reliability of our microarray results.

IL-1A and IL-1B inhibit hair growth in vitro as published previously [20]. However, IL-1B has shown upregulation at the August time point in our study. Wool was supposed to grow faster during August than in December.

A log linear linkage exists between the relative level of *ITGB1* on the cell surface and proliferative capacity in keratinocytes [23]. *ITGB1* signalling is also essential for human hair growth [24]. Skin and hair follicle integrity is

crucially dependent on *ITGB1* expression in keratinocytes [25]. In our study, however, *ITGB1* expression in A/D was down-regulated. And *ITGB1* level was also lower in wool bearing area and in wool-less area in another study (data not shown). The reasons how those happen deserve further investigation.

During development process, *GluD1* was distributed to the hippocampus, cochlear and vestibular hair cells, and spiral ganglion cells [26–28]. Hair follicles are also derived from ectoderm. So why *GLUD1* showed down-regulated at the transcription level in A/D remains to be further investigated.

*Connexin 43* is a gap junction protein distributed in the follicular dermal papilla [28]. A recent study by mouse model suggested *Connexin 43* plays vital roles in hair regeneration, growth, and cuticle formation [29]. However, *Connexin 43* displayed downregulation in A/D in the transcriptional level of our study, and it was subsequently validated by real time qPCR.

We found that a high percentage of DE genes play important roles during embryo development. As displayed in Table S2 (Supplementary material), 3 pregnancy-associated glycoproteins (*PAG 6*, *PAG 9* and *PAG 11*) were up-regulated in A/D. Furthermore, we found dozens of immune-associated genes from the DE gene list, such as heat shock proteins, interleukins, matrix metalloproteinases and clusters of differentiation. Similar phenomena were found in another study (data submitted to BMC Genetics). The immune privilege mechanism exists both in hair follicle cycling and embryo development. Therefore, in a sense, hair follicles revealed similar features with the embryo, and that the regulation mechanism of hair follicle might resemble that of fetation.

#### Significance of differentially expressed proteins in the proteomic study

Eighty-four protein spots were differentially expressed. Of the 84, 63 protein spots were upregulated and 21 were



downregulated in A/D. 55 protein points were determined through MALDI-TOF/MS analyses. Most of the DE proteins were not discussed above.

Cryab protein was detected in 2DE in August time point but not in December time point. However, the Cryab transcripts show no differential expression in the microarray level. This gene, downregulated in the transcriptional level during the depilation-induced hair cycle [30], was considered to play a role in regulation of apoptosis. So our results support this inference.

KRT5, KRT14, KRT2.11 (KRT86) and KRT19 proteins were all upregulated in A/D. These proteins were reported to play important roles in wool follicles [31–35]. KRT14 and KRT19 are type I keratin, while KRT2.11 (KRT86) and KRT5 are type II keratin [16, 32]. In secondary versus primary follicles of Yunnan black goat, the two differentially expressed keratin genes (keratin 40 and 72) were type I and type II, respectively [16]. So similar mechanism or related biological process might exist between wool cycling and cashmere development.

*FGFR3* RNA distributed in precuticle cells in the periphery of the hair bulb [36, 37]. Consistently, FGFR3 protein was upregulated in August time point compared to the control. We could deduced that FGFR3 may play positive roles in wool growth.

Concordance between the results of transcriptomic and proteomic levels

In the results of transcriptomic level (microarray), the number of up-regulated genes was higher than that of down-regulated ones (1,162 vs. 1,061). Consistently, up-regulated protein spots were more than down-regulated ones (63 vs. 21).

The concordance between these two results from microarray and proteomic experiments was shown in Table S4 (Supplementary material). Of the 55 identified proteins, 5 corresponding transcripts also shown differential expression. Among these 5 transcripts (CNN1, HSP70.1, OVGPI, TYR and Flt-1), the regulation trends of 4 were consistent with their protein counterparts except HSP70.1. The inconsistency of the two levels of HSP70.1 expression might be produced by post transcriptional regulation.

## Conclusion

In summary, the results in this research suggested that the body side skin at August time point displayed a differentially expressed pattern in comparison with that at December time point. Most of these DE genes were supposed to be associated with the wool growth cycling regulation. The majority of the genes possibly related to

follicle development and wool growth could be assigned into the categories including regulation of receptor binding, extracellular region, protein binding and extracellular space. Several gene families of growth factors might take part in hair growth regulation, including fibroblast growth factors, transforming growth factor- $\beta$ , insulin-like growth factor, and so on. Proteomic analysis also identified dozens of differentially expressed proteins. This whole genomic study may shed light on mechanism of the wool growth cycling control in Aohan fine wool sheep.

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