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Label-free proteomics to identify keratins and keratin-associated proteins and their effects on the fleece traits of Inner Mongolia Cashmere Goats

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Abstract: Inner Mongolia Cashmere Goat fleece is derived from a heterogeneous coat. The guard hair is produced by primary hair follicles, and the down hair is produced by secondary hair follicles. Reports have focused on research related to the different hair follicle types, but no related study has addressed the difference in the proteins of down hair (DH) and guard hair (GH), and whether the protein composition of these materials differs remains to be studied. The protein composition of DH and GH was studied. A total of 108 proteins were identified in DH, and 116 proteins were identified in GH; 39 proteins were differentially expressed, of which 18 proteins were upregulated and 21 proteins were downregulated in DH versus GH. In this study, through the measurement of DH and GH samples from Inner Mongolia Cashmere Goats, the diameter of DH relative to GH was downregulated ($P < 0.05$), a property that may be related to the KRT38 protein. Moreover, the strength of GH was significantly higher than that of DH ($P < 0.05$), a property that may be affected by the KRTAP8-1 protein. GO analysis showed that the different traits of DH and GH from Inner Mongolia Cashmere Goats are affected by keratin, actin, and calcium-binding proteins. This study uses a nonlabelled quantitative proteomics method to study the proteins in DH and GH, aiming to identify the keratin family in Inner Mongolia Cashmere Goats and to provide a new direction for studying DH and GH traits at the protein level.

Keywords: KRT1; KRT38; KRTAP8-1; strength; average diameter

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As the largest producer of down hair (DH) worldwide, China accounts for 50% of the global output of DH, of which DH production by Inner Mongolia Cashmere Goats accounts for approximately 30% (Duan et al. 2015). Cashmere goat hair follicles can be divided into primary hair follicles and secondary hair follicles (Dong et al. 2013). Guard hair (GH) fibres grow from primary hair follicles and are thick and long with a medullary layer; down hair fibres grow from secondary hair follicles and are short and thin without a medullary layer (Ji et al. 2016). Cashmere goats provide heterogeneous fleece and are a good model for studying differences in cashmere formation.

Proteins are the basis of animal fibres, and the protein content in animal fibres can be as high as 90–95%. The main proteins in DH and GH are keratins (KRTs) and keratin-associated proteins (KRTAPs). Proteomic studies on fibre composition have focused mainly on human hair (Langbein and Schweizer 2005), and a total of 25 KRTs have been identified in human hair (Rogers et al. 2006). Both KRTs and KRTAPs are embedded in the related matrix by keratin intermediate filaments and crosslinked by interchain disulfide bonds during keratinization. KRTs and KRTAPs play an important role in the structure of DH and GH. A study by Wu and Irwin (2018) showed that *KRTAP* gene variants have an important effect on the breeding of sheep and on DH fibre and that the high glycine-tyrosine KRTAP is related to GH crimps. KRTs and KRTAPs strongly influence fibre traits. For example, variations in the *KRTAP6* gene are related to GH fibre diameter (Parsons et al. 1994), and variations in the nucleotide sequences of *KRTAP8-2* (Niimura and Nei 2007), *KRTAP13-1* (Wang et al. 2017), *KRTAP15-1* (Niimura and Nei 2003), *KRTAP20-1* (Grus et al. 2007), *KRTAP20-2* (Grus et al. 2005), *KRTAP24-1* (Niimura and Nei 2005) and *KRTAP28-1* (Niimura and Nei 2006) are related to the traits of DH in goats. KRTs and KRTAPs also affect the length and weight of curled fibres of GH and DH. Variations in *KRTAP20-2* (Wang et al. 2017), *KRTAP8-2* (Liu et al. 2007), and *KRTAP13-1* (Fang et al. 2010) affect the length and quality of DH and GH fibres. In addition, KRTs and KRTAPs are involved in the growth of DH and GH fibres. *KRTAP9-2* may be related to DH growth (Wang et al. 2012), but *KRTAP7-1* and *KRTAP8-2* are involved in GH formation (Jin et al. 2011). KRTs and KRTAPs have a profound influence on the characteristics of DH and GH.

Protein composition, qualitative and quantitative analysis, structure, and functional data can be obtained through proteomic analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be used to determine the sequence of amino acids and the relative molecular masses of proteins and peptides with high accuracy. This technique is used in differential proteomics, quantitative protein analysis, and protein analysis. The ability of this technique to identify protein-protein interactions also has great advantages. Therefore, in this study, we used LC-MS/MS to quantitatively analyse the proteome of Inner Mongolia Cashmere Goats to identify the KRTs that affect the traits of DH and GH. This research lays a foundation for improving DH and GH quality. Moreover, it establishes a theoretical basis and provides data support for authentication and component analysis of DH and GH.

MATERIAL AND METHODS

Fibre samples

DH and GH were sampled from each animal (two years old, carcass weight of 25–26 kg) to obtain 30 replicate samples. All the samples were obtained from Inner Mongolia Cashmere Goats (Alpas, Yiwei White Down Hair Goat Breeding Farm, Erdos, Inner Mongolia), and the procedures were approved by the Experimental Animal Ethics Committee of Inner Mongolia Agricultural University (GB 14925-2001). The hair of each goat was cut close to the skin to collect 5 g of body side hair, and the amount of hair cut was recorded. The DH and GH samples were collected aseptically in enzyme-free tubes and subsequently stored at –20 °C until analysis.

Measurement of economically important parameters

The natural length, diameter, and strength of the DH and GH fibres were measured in the laboratory; the structures of DH and GH were visualized using an EVO10 scanning electron microscope (ZEISS, Shanghai, China) at different magnifications (fibre structure was analysed basically according to International Wool Textile Organization standard IWTO-58-00). Samples were washed, dried and

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tested under conditions of constant temperature and humidity (20 ± 2 °C, 65 ± 4%). The complete raw hair tuft was obtained at the natural length, and the DH and GH samples were measured separately, taking care to maintain the original shape of the hair bundle. A steel ruler was used to measure the natural length of the hair samples under normal temperature and humidity conditions. The length was measured according to the GB/T6976-2007 standard (Test Method for the Natural Length of Guard Hair Floss). The diameter was measured using an OFDA 2000 (Apek International, Hong Kong, China) optical fibe diameter analyser according to the GB/T21030-2007 standard (Guard Hair and Other Animal Fibre Average Diameter and Distribution Test Method, Fibre Optical Diameter Analyzer Method). Hair strength was measured with a YG006 Electronic Single Yarn Strength Tester (Bohui Instrument, Xi'an, China) according to the GB/T3916-1997 standard (Spinning Products, Package Single Yarn Breaking Strength and Breaking Elongation).

Protein extraction

The DH (0.5 g) and GH (0.5 g) samples were rinsed with water. Then, 1 ml of a dichloromethane and methanol mixture was added to each sample, and the samples were shaken at 50 °C for 2 hours. The samples were centrifuged and then dried at 50 °C. The samples were placed into a pyrolysis buffer containing protease inhibitor (Roche, Basel, Switzerland) and 1% sodium dodecyl sulphate (Kulabor, Shanghai, China) and then placed in an oven at 37 °C for 48 hours. The samples were then removed from the oven and centrifuged at 1 000 rpm at 4 °C for 20 minutes. The supernatants were collected for further study, and the protein concentrations were measured with a bicinchoninic acid (BCA) kit (Tiangen, Shanghai, China).

Tryptic digestion of proteins

The procedure was performed basically according to Xie et al. (2022). To the samples of protein solution (see above), containing 100 µg of protein, 200 µl of 8 M urea and 10 mM DL-dithiothreitol were added and incubated at 37 °C for 1 hour. After centrifugation at 12 000 rpm for 40 min, the precipitate was retained, and the supernatant was discarded. Then 200 µl of 8 M urea was added to the precipitate and stirred. The tubes were centrifuged twice at 12 000 rpm for 30 min, and after each centrifugation the supernatant was discarded. Then, 200 µl of 50 mM iodoacetamide was added to each tube and allowed to stand in the dark for 30 min, centrifuged at 12 000 rpm for 10 min, and the supernatant was discarded. Then, 100 µl of 100 mM ammonium bicarbonate was added to each tube, and the samples were centrifuged at 12 000 rpm for 20 minutes. This step was performed three times, and after each centrifugation the supernatant was discarded. The samples were incubated overnight with 4.3 µl (2.15 µg) trypsin (Promega, Madison, USA) at 37 °C and centrifuged at 12 000 rpm for 30 minutes. The supernatant was collected and the precipitate was discarded. Then, 50 µl of 100 mM ammonium bicarbonate was added to each tube. The samples were centrifuged at 12 000 rpm for 30 min, the supernatant was collected, and this step was repeated. The supernatant was freeze-dried and stored at –20 °C.

LC-MS/MS analysis

In the LC-MS/MS system (Sciex, Framingham, MA, USA), information-dependent capture (IDA) and sequential window acquisition of all theoretical spectra-mass spectrometry (SWATH-MS) were used to obtain peptide data. Approximately 2 µg of polypeptide was separated by injection on a C18 column (75 µm × 15 cm). The peptides were separated by a linear gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (120 min, from 5% to 80%, 500 nl/min). The IDA conditions were as follows: nominal resolution, 30 000; time-of-flight mass spectrometry acquisition range, 350–1 800 m/z; MS/MS and IDA scan range, 400–1 800 m/z; automatic collision energy. The SWATH-MS conditions were as follows: 150–1 200 m/z, MS1 mass range; 100–1 500 m/z, MS2 mass range; nominal resolutions of MS1 and MS2, 30 000 and 15 000, respectively.

Protein identification and difference analysis

Protein Pilot v4.5 software (Sciex, Framingham, MA, USA) and the UniProt/SWISS-PROT/*Capra*

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hircus database (https://www.UniProt.org/#) were used for peptide identification. The results were filtered with a 1% false discovery rate (FDR). The selected search parameters included the use of trypsin as the enzyme, allowing up to two missed cleavage sites. The peptide mass tolerance was ± 15 ppm, and the fragment mass tolerance was 20 mmu. The data were loaded into PeakView (Sciex, Framingham, MA, USA) software, and the ion library generated by Protein Pilot was used to search the SWATH database. PeakView processed the target and nontarget data to generate the extracted ion chromatograms (XICs). Then, MarkerView software (Sciex) was used to explain and quantitatively analyse the results. MarkerView allows a rapid review of data to determine the differentially expressed proteins (DEPs). Principal component analysis (PCA) and volcano plot analysis, which combined the fold change analysis and *t*-tests, were performed. A fold change > 2 or fold change < 0.5 and statistical significance (*P*-value < 0.05) were used to identify DEPs (Ebhardt et al. 2017).

Bioinformatics analysis

The proteins were subjected to bioinformatics analyses. The g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) online software was used to perform the Gene Ontology (GO) analyses. STRING (https://cn.string-db.org/) was used to perform the protein-protein interaction (PPI) analysis. SWISS-MODEL (https://swissmodel.expasy.org/) was used to predict the three-dimensional structure of the proteins (Xie et al. 2022).

Western blotting

The source proteins obtained above were boiled at 100 °C for 3 min to become denatured proteins. The procedure was referenced according to Xie et al. (2022). The denatured proteins were separated using SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Lincoln, NE, USA). PVDF membranes were incubated overnight in a dilution of mouse monoclonal anti-rabbit KRT1 primary antibody (Abcam, Cambridge, MA, USA; diluted 1 : 1 000). The membranes were incubated with fluorescent goat anti-mouse secondary antibody (LI-COR Biosciences, Lincoln, NE, USA; dilution 1 : 30 000) for 1 h after washing three times. The results were finally observed with a LI-COR Odyssey 10 NIR imager (LI-COR Biosciences).

The same denatured proteins (as above) were separated using SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Actin beta expression was similar in DH and GH, so it was chosen as reference protein. PVDF membranes were incubated overnight in a dilution of mouse monoclonal anti-rabbit actin primary antibody (Abcam; diluted 1 : 1 000). The membranes were incubated with fluorescent goat anti-mouse secondary antibody (LI-COR Biosciences; dilution 1 : 30 000) for 1 h after washing three times. The results were finally observed with a LI-COR Odyssey 10 NIR imager (LI-COR Biosciences).

RESULTS

Determination and analysis of the traits of down hair and guard hair

To analyse the structural differences between DH and GH, 30 samples of DH and GH were obtained from three cashmere goats for length, diameter, and strength measurements and visualization of the scale structure. Each index was repeatedly measured three times. The average lengths of the Inner Mongolia Cashmere Goat DH and GH fibres were 8.91 cm and 7.60 cm, respectively; the average diameter values were 16.45 μm and 43.06 μm, respectively; and the average elongation rates at breakage were 44.38% and 52.01%, respectively. The GH diameter was significantly higher than the DH diameter ($P < 0.05$), and the average strengths were 6.9 cN/dtex and 50.03 cN/dtex, respectively; the GH strength was significantly higher than the DH strength ($P < 0.05$) (Table 1). The results of electron microscopy indicated that the scale length of DH was greater than that of GH, that the scale number and density of GH were greater than those of DH (Figure 1), and that the fibre uniformity and glossiness of DH were higher than those of GH. Electron microscopy allowed visualization of the differences in the surface structure of DH and GH fibres, and proteomic analysis can identify the main reason for the differences in the surface structure.

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Table 1. Statistics of down hair and guard hair traits of Inner Mongolia Cashmere Goats

Traits	Length (cm)		Diameter (μm)		Strength (cN/dtex)		Elongation at break (%)	
Category	DH	GH	DH	GH	DH	GH	DH	GH
Average	8.91	7.60	16.45	43.03	6.90	50.03	44.38	52.01
Standard deviation	1.05	1.81	0.23	5.51	2.30	12.96	5.58	6.81
Coefficient of variation (%)	11.78	23.81	1.40	12.81	33.33	25.90	12.57	13.09

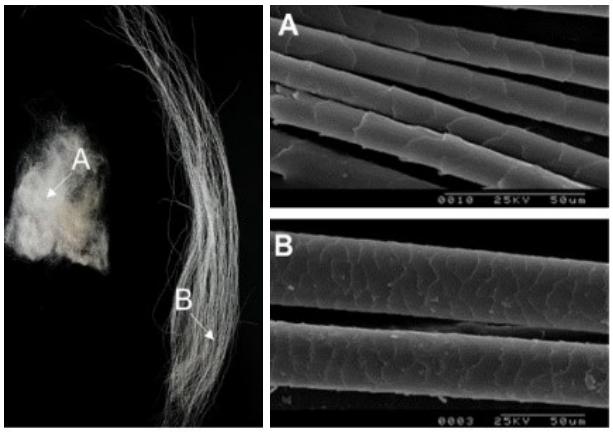

Figure 1. Scanning electron micrographs
(A) Down hair fibres; (B) guard hair fibres; magnification 1 000×

Analysis of protein expression in down hair and guard hair

The total protein content of DH and GH was quantitatively analysed against the UniProt/Swiss-Prot/*Capra hircus* database. With the criterion FDR ≤ 0.01, a total of 141 proteins were identified in DH and GH. In DH, 25 KRTs, 4 KRTAPs, and 79 other proteins were identified. In GH, 31 KRTs, 3 KRTAPs, and 82 other proteins were identified.

For a more clear survey, the identified proteins are categorized below. KRTs and KRTAPs are specified.

Proteins that were expressed both in DH and GH: 73 proteins, of which were 16 KRTs (KRT14, KRT17, KRT75, KRT38, KRT33A, KRT32, KRT15, KRT84, KRT35, KRT85, KRT82, KRT40, KRT5, KRT77, KRT10, KRT1) and 4 KRTAPs (KRTAP13-3, KRTAP15-1, KRTAP19-3, KRTAP13-1); proteins that were expressed only in DH: 35 proteins, of which were 2 KRTs (KRT80; keratin, type I, microfibrillar, 47.6 kDa) and 2 KRTAPs (KRTAP16-2; KRTAP8-1); proteins that were expressed only in GH: 33 proteins, of which were 3 KRTs (KRT79; KRT3; KRT39); proteins that were upregulated in DH: 18 proteins, of which were 4 KRTs (KRT14; KRT17; KRT75; KRT38); proteins that were downregulated in DH: 21 proteins, of which were 5 KRTs (KRT5; KRT77; cytokeratin-1; KRT10) and 1 KRTAP (KRTAP13-1).

All identified proteins are presented in Table S1 in electronic supplementary material (ESM; for the ESM see the electronic version), and upregulated and downregulated proteins are shown in Table 2. To classify the keratin protein family in goats, we built a phylogenetic tree using all 33 keratin protein (https://www.uniprot.org/) sequences from the cashmere goat using the neighbour-joining method. This tree illustrated that the KRTs in DH and GH in the cashmere goat can be divided into four subfamilies (Figure 2B).

Analysis of differential protein expression between down hair and guard hair

Differential protein expression analysis of DH and GH was carried out, and the results are shown in Table 2 and Table S1 in ESM. The quantifiable proteins extracted after mass spectrometry analysis were imported into MarkerView software (Sciex, Framingham, MA, USA) for PCA. The results showed the similarities and differences between DH and GH in score plots; 63.5% of the variability was explained by principal components 1 and 2, which accounted for 39.6% and 23.9% of the total variance, respectively (Figure 3A). Samples from the two groups were separated and located in different quadrants, indicating the existence of DEPs between DH and GH. A total of 39 DEPs were identified with threshold criteria of $P < 0.05$ and fold change > 2 or fold change < 0.5. These proteins included 10 types of KRTs, two types of KRTAPs, and 27 types of other proteins.

In the comparison between DH and GH, 18 proteins were upregulated, and 21 proteins were downregulated (Table 2, Figure 3B). (The designation upregulated and downregulated concerns DH versus GH throughout the manuscript).

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Table 2. Main differential proteins affecting the properties of down hair (DH) and guard hair (GH) in Inner Mongolia Cashmere Goats[1]

Accession number[2]	Gene symbol	Protein name	Fold change[3]	*P*-value
A0A452EME0	*KRT14*	keratin 14	< 0.001	20.826
A0A452FL58	N/A	IF rod domain-containing protein	0.032	16.446
K9LQQ8	*FABP3*	fatty acid-binding protein, adipocyte	< 0.001	13.578
A0A452E4U1	*KRT17*	keratin 17	< 0.001	10.630
A0A452EML6	*KRT75*	keratin 75	< 0.001	10.170
A0A452DSB1	*ANXA2*	annexin	< 0.001	7.905
A0A452FE14	*LOC108633262*	IF rod domain-containing protein	< 0.001	7.258
A0A452DXH0	*CDSN*	corneodesmosin	< 0.001	6.179
A0A452FGV2	*CAPG*	macrophage-capping protein	< 0.001	3.195
H9B8T8	N/A	peroxiredoxin 2	< 0.001	3.092
A0A452FI49	*LOC102169411*	secretoglobin family 1D member 1	0.008	3.070
A0A452EBY7	*KRT38*	IF rod domain-containing protein	0.019	3.015
B3VHM9	N/A	albumin	0.180	2.858
A0A452F0W7	*TPM3*	tropomyosin 3	0.033	2.450
A0A452EH63	*ALDOA*	fructose-bisphosphate aldolase	< 0.001	2.376
A0A452FN70	*BANF1*	BAF nuclear assembly factor 1	0.023	2.182
A0A452E1U2	*CALML5*	calmodulin like 5	0.049	2.130
A0A452ERK8	*SRI*	sorcin	0.007	2.086
A0A452FEQ1	N/A	EF-hand domain-containing protein	0.017	0.467
A0A452F1P5	*RPS28*	40S ribosomal protein S28	< 0.001	0.456
A0A452E8H3	N/A	HMA domain-containing protein	0.003	0.432
A0A452DMF8	*KRT5*	keratin 5	< 0.001	0.425
A0A452FKL0	*DSC3*	desmocollin 3	0.020	0.421
A0A452FF94	*GLRX*	glutaredoxin domain-containing protein	< 0.001	0.389
A0A452ELA7	*KRT77*	keratin 77	< 0.001	0.381
A0A452E969	N/A	major allergen I polypeptide chain 1-like	0.002	0.359
A0A452G9K5	*KRT1*	keratin, type II cytoskeletal 1	< 0.001	0.354
Q95L76	*CSN2*	casein beta	0.093	0.345
A0A452EKA5	*MLF2*	myeloid leukemia factor 2	0.002	0.344
F6KVT3	*sTNC*	fast twitch skeletal muscle troponin C2	0.017	0.336
A0A452E0C3	*HSPE1*	chaperonin 10	0.035	0.325
A0A452GAV6	*LOC102176161*	IF rod domain-containing protein	< 0.001	0.316
A0A452G0P6	*KRT10*	keratin 10	0.001	0.249
A0A452ECK9	*LOC102181431*	keratin-associated protein 13-1-like	< 0.001	0.241
A0A452GA47	*KRT1*	cytokeratin-1	< 0.001	0.237
A0A452EXF9	N/A	histone H2A	0.002	0.188
A0A452FD11	*TPM1*	tropomyosin 1	0.046	0.183
A0A452DPJ6	*MYLPF*	myosin light chain, phosphorylatable, fast skeletal muscle	0.020	0.125
A0A452FKU3	*S100A7A*	protein S100	0.010	0.066

N/A = not applicable

[1]The upregulated proteins in DH are above the line in the table and the downregulated proteins in DH are below the line

[2]Accession number comes from the UniProt/SWISS-PROT/*Capra hircus* database (https://www.UniProt.org)

[3]Fold change is used to describe the degree of change from an initial value to a final value. *P*-value mainly reflects the difference between groups, and its size represents the strength of the difference in evidence. Upregulated proteins and downregulated proteins can be judged according to their values

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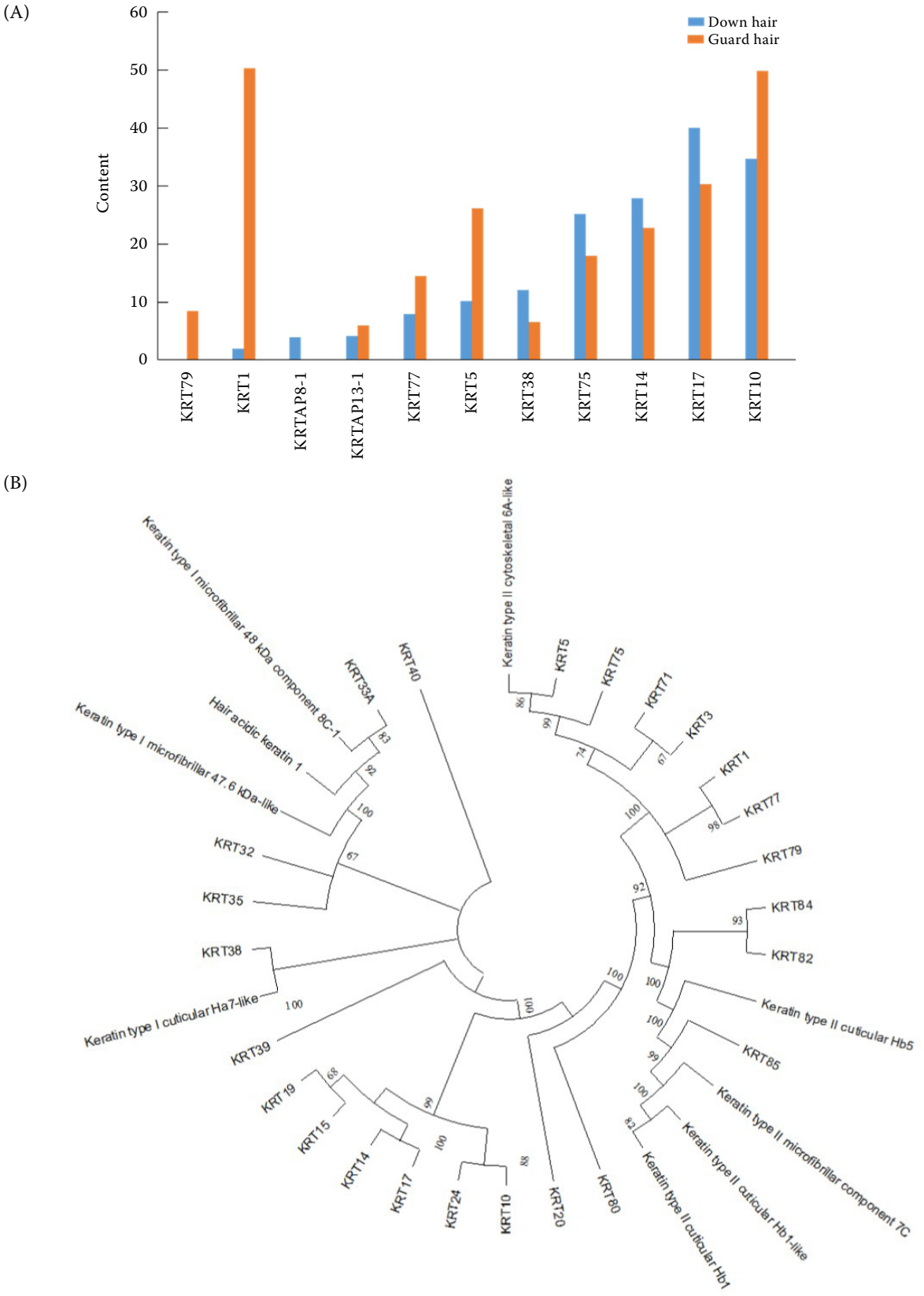

Figure 2. Main differentially expressed proteins affecting the properties of down hair and guard hair in Inner Mongolia Cashmere Goats

(A) Expression of keratin proteins in down hair and guard hair; (B) phylogenetic tree of goat keratin proteins

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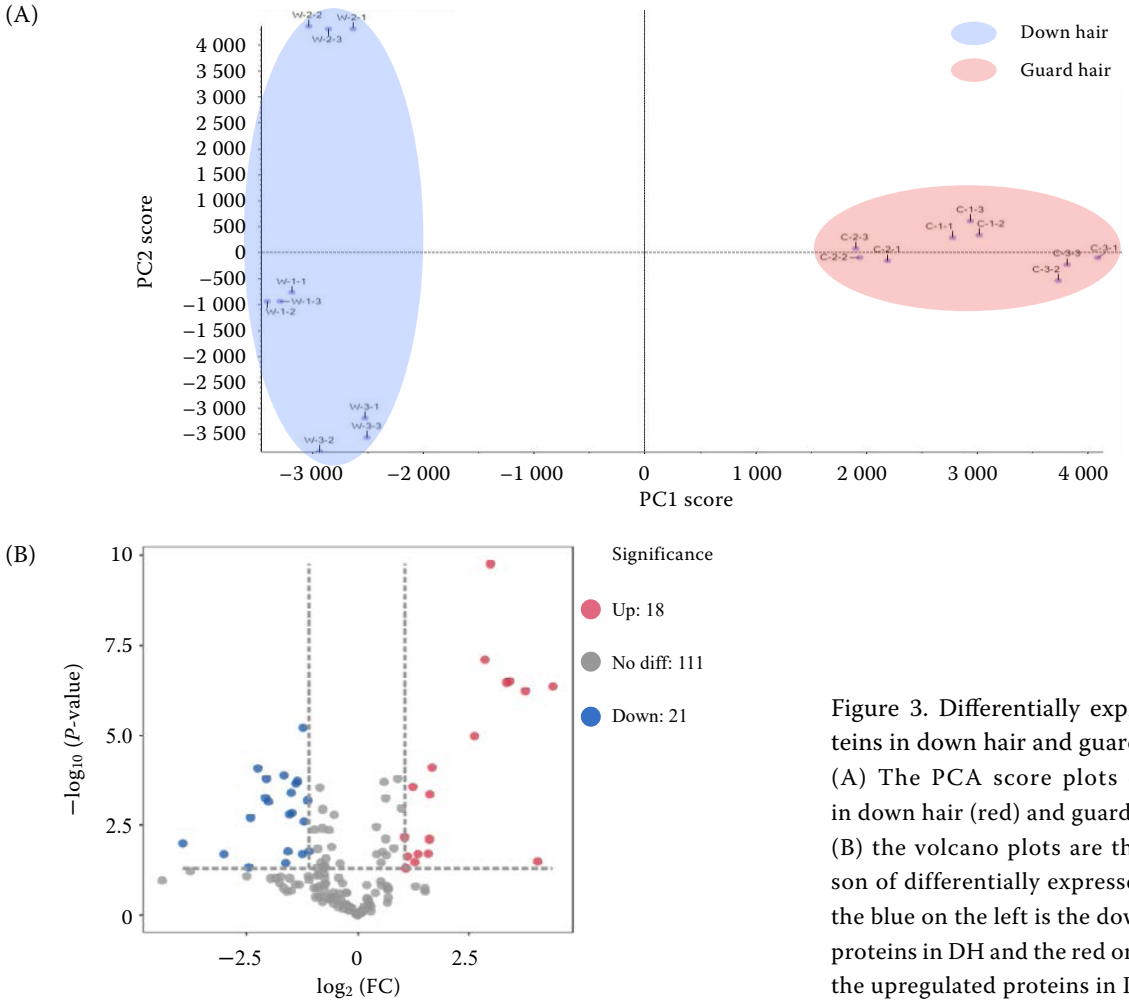

Figure 3. Differentially expressed proteins in down hair and guard hair
(A) The PCA score plots of proteins in down hair (red) and guard hair (blue); (B) the volcano plots are the comparison of differentially expressed proteins, the blue on the left is the downregulated proteins in DH and the red on the right is the upregulated proteins in DH

Functional analysis of differentially expressed proteins

GO functional analysis of the DEPs showed that they were enriched mainly in the terms intermediate filament, supramolecular fibre, polymeric cytoskeletal fibre, keratin filament, and structural constituent of skin epidermis (Figure 4). All of these are related to the fibre structure. To date, four proteins have been confirmed to play an important role in DH or GH fibres (CALML5, LOC102176161, KRT38, KRTAP8-1) (Zheng et al. 2019).

Analysis of protein-protein interaction networks

In this study, PPI networks were constructed using STRING software. Interactions were found between 19 of the 30 proteins, the number of edges was 55, and the average local clustering coefficient was 0.503 for the different proteins. KRT1 was connected to KRT5, KRT77, KRT38, KRT10, KRT75, KRT14, KRT79, KRT17, and CALML5 (Figure 5). A three-dimensional analysis of the structure might provide an insight into the relationship between the structure and function of KRT1 and KRT38 in future studies (Figure 6).

Western blot analysis of KRT1 expression

Among all the DEPs, KRT1 was the most highly expressed, and the PPI network analysis showed that it was strongly correlated with other KRTs. Thus, the KRT1 protein was screened by Western blot analysis (Figure 7A). The expression of KRT1 in GH was significantly higher than that in DH ($P < 0.05$) (Figure 7B). This pattern was consistent with the quantitative proteomics results, further indicating the accuracy of the proteomics results.

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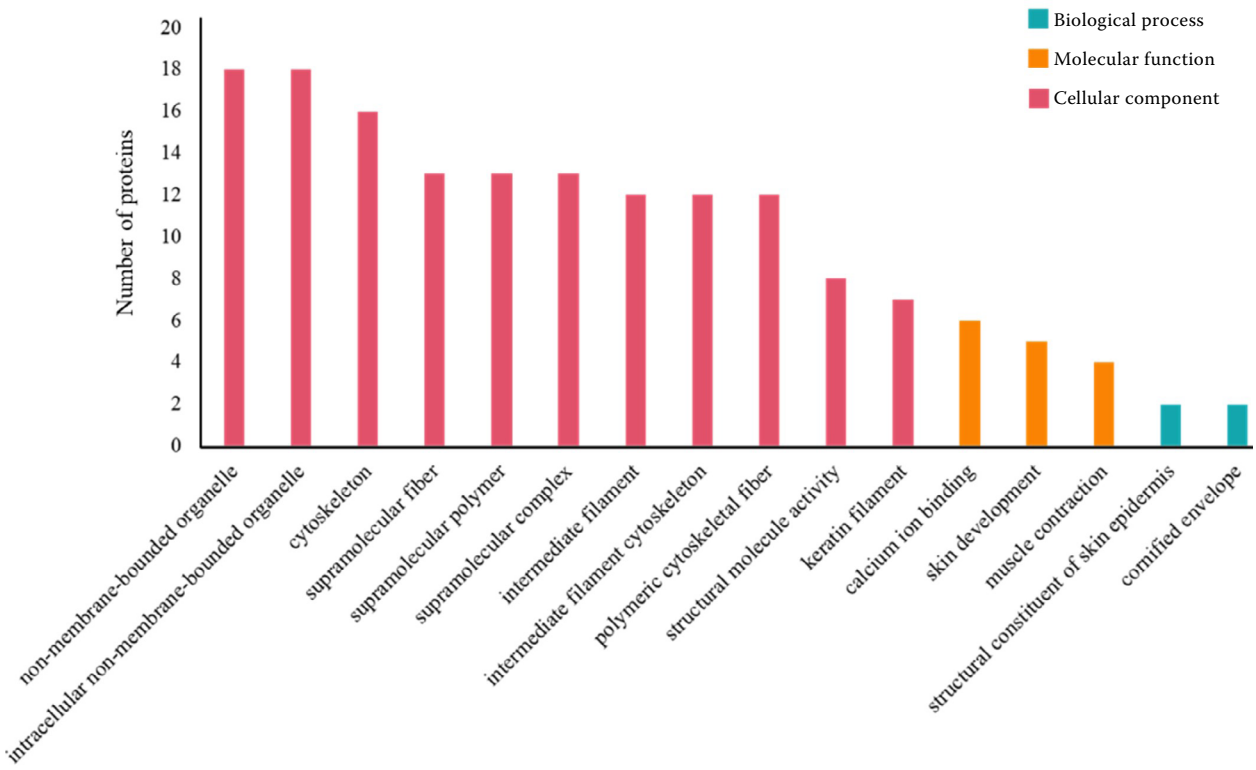

Figure 4. GO functional analysis of down hair and guard hair different proteins in Inner Mongolia Cashmere Goats
The *x*-axis represents biological categories; the *y*-axis represents the number of proteins in particular categories

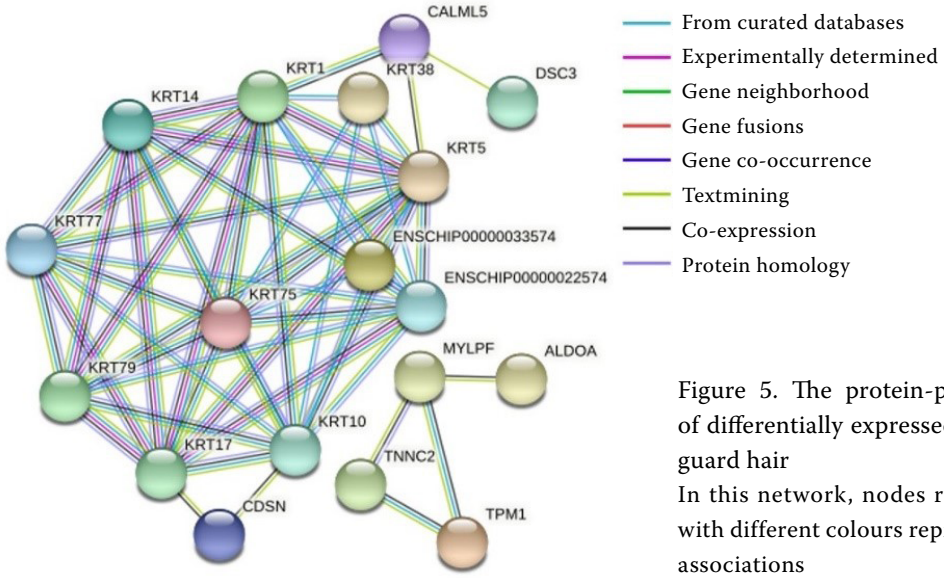

Figure 5. The protein-protein interaction network of differentially expressed proteins in down hair and guard hair
In this network, nodes represent proteins, and lines with different colours represent the predicted different associations

DISCUSSION

The cashmere goat is very important to economic production and human lifestyle. The quality and value of textiles depend largely on the colour, diameter, length, fibre source, and availability of DH. A study of hair samples from sheep and goats showed that the two sample types shared 173 proteins, including 28 KRTs or KRTAPs (Li et al. 2018). By using the LS-MS/MS, LC-MALDI, 2D LC-MS/MS, and SDS-PAGE LC-MS approaches, Clerens et al. (2010) studied the GH proteome and identified 113 proteins in GH, adding 72 complete and 30 partial ovine-specific protein sequences to the

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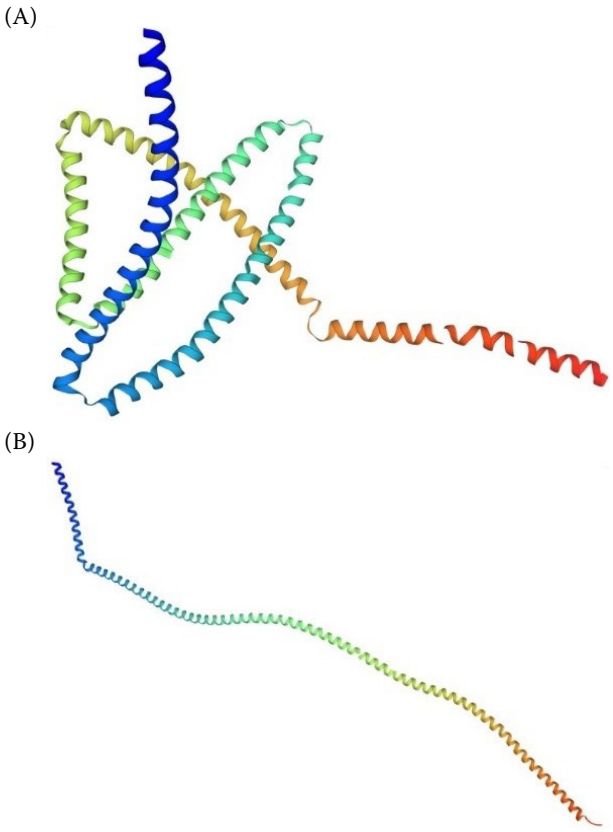

Figure 6. Three-dimensional structure of the protein
The three-dimensional structure of the protein is predicted based on the amino acid sequence (A) three-dimensional structure of KRT1; (B) three-dimensional structure of KRT38

database. In the present study, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was used to study the proteomes of DH and GH from Inner Mongolia Cashmere Goats, and 141 proteins, including 17 KRTs and 5 KRTAPs, were found to be shared by DH and GH. In DH, 25 KRTs, 4 KRTAPs, and 79 other proteins were identified. In GH, 31 KRTs, 3 KRTAPs, and 82 other proteins were identified.

Proteins do not function independently but perform various functions through interactions with each other. Moreover, KRTs related to the structure of DH and GH (KRT1, KRT5, KRT10, KRT14, KRT17, KRT75, KRT77, KRT79, and KRT75), proteins related to DH and growth (DSC3 and CALML5), proteins involved in fatty acid synthesis (FABP3) and other proteins that affect the structural differences between DH and GH, such as S100A7A, MYLPF, TPM1, TPM3, LOC102169411, LOC102181431, KRTAP8-1, HSPE1, sTNC, MLF2, GLRX, RPS28, SRI, BANF1, ALDOA, LOC102176161, CAPG, CDSN, LOC108633262 and ANXA2, were identified.

KRTs and KRTAPs are the main structural proteins of the hair sheath and hair fibre, and their levels have an important impact on the quality of DH and GH (Giesen et al. 2011), including on strength and diameter. These proteins play a key role in the formation of strong hair shafts, and their levels have an important influence on the quality of DH and the shape of GH (Giesen et al. 2011). With the continuous improvement of DH output and quality, the diameter and quality of DH have begun to be considered. Some proteins that affect the composition and structure of DH and GH have been identified, and most of these proteins belong to the KRT family. Understanding the structure and characteristics of fibres is important. The KRTAP8-1 protein was first reported in sheep and was the first high glycine-tyrosine keratin-associated protein to be discovered. In Southdown × Merino crossbred lambs, KRTAP8-1 mutation has a moderate short-term effect not only on guard hair but also on fibre strength (Gong et al. 2019). In this study, when the DH and GH samples were tested, the average strengths were 6.9 cN/dtex and 50.03 cN/

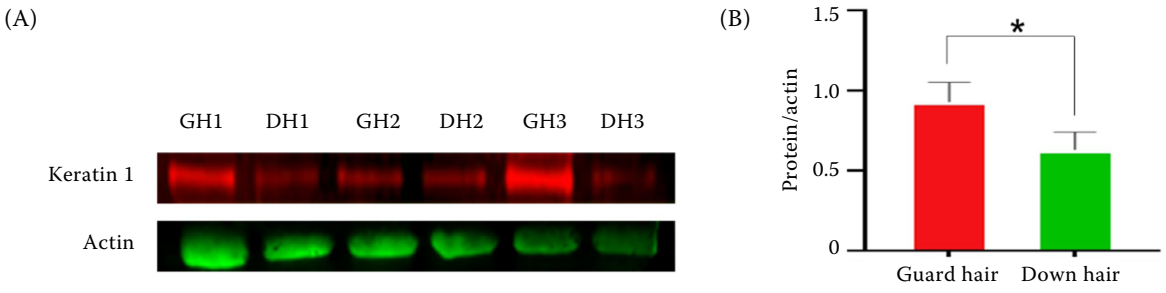

Figure 7. Western blot profiles of KRT1
(A) Three biological replicates from the tissue of down hair (DH) and guard hair (GH); (B) relative intensity of protein is expressed as the mean ± standard deviation. Asterisk represents levels of significance (*t*-test: $^*P < 0.05$)

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dtex, respectively. The strength of GH was significantly higher than that of DH ($P < 0.05$). KRTAP8-1 is highly expressed in DH, but it is not expressed in GH. Therefore, we predict that KRTAP8-1 is a key candidate protein that affects DH and GH fibre strength.

KRT38 is considered a key candidate protein affecting the diameter of DH (Yu et al. 2011). Moreover, KRT38 expression has been found to differ between DH and GH from cashmere goats (Zheng et al. 2019). In this study, the average diameters of DH and GH were 16.45 μm and 43.06 μm, respectively, as determined by measuring the DH and GH samples. The diameter of GH was significantly higher than that of DH ($P < 0.05$), and proteomic analysis was used to identify the proteins contributing to the diameter of DH and GH from Inner Mongolia Cashmere Goats. Among the DEPs, KRT38 showed a trend toward upregulation. Therefore, we believe that KRT38 is the key protein mediating the difference between DH and GH.

The selection of proteins by LS/MS-MS analysis was based on our understanding of the relationship between fibre morphology and KRT content. Keratinization is an example of controlled cell apoptosis. Most proteins in fibres are removed, leading to the dominance of KRTs. This was further supported in this study. The major KRTs and KRTAPs (Deb-Choudhury et al. 2010; Plowman et al. 2019) identified in other studies had the highest sequence coverage, while nonkeratins had the lowest sequence coverage, most being identified with only one peptide. In our study, the sequence coverage of KRTAP13-3, KRTAP15-1, and KRT33A ranged from 90% to 99%. In contrast, the nonkeratinous proteins had a very low sequence coverage; for example, TMEM150C had a sequence coverage of 4%, and CCNT2 had a coverage of 8.1%. These results provide further support for the proteomics data, the most important of which is related to the medullary level of the two fibre types. In particular, the content of type II keratin 75 found in the GH fibre medulla (Langbein et al. 2010) was twice that in down hair. The average fibre diameters of GH and DH were significantly different ($P < 0.05$). This suggests that the protein may be a good marker for determining the extent of *medulla oblongata* retraction in goats.

The research provides a theoretical basis for the identification of DH and GH, provides data support for the future improvement of DH and GH quality, and lays a foundation for the further development and utilization of the economic value of Inner Mongolia Cashmere Goats. An unexpected result of this study was the discovery of the potential of the LS/MS-MS method to distinguish between different fibres in goats. Although the sample size was limited to three, DH and GH could be distinguished.

CONCLUSION

In this study, a total of 141 proteins were identified in the fibre of Inner Mongolia Cashmere Goats, among which 116 proteins were identified in DH and 108 proteins were identified in GH. Through the measurement of DH and GH samples from Inner Mongolia Cashmere Goats, the diameter of GH was found to be significantly higher than that of DH ($P < 0.05$), a property that may be related to the KRT38 protein. Moreover, the strength of GH was significantly higher than that of DH ($P < 0.05$), which may be affected by the KRTAP8-1 protein. This study provides a useful reference for further understanding the relationship between KRT/KRTAP and fibre traits. The following studies need to further determine the relationship between KRT38/KRTAP8-1 and fibre traits, its regulatory effect on the growth and development of down hair, which is of great significance for the in-depth understanding of the mechanism of down hair and guard hair growth and development of goats.

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Conflict of interest

The authors declare no conflict of interest.

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