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RESEARCH ARTICLE

iTRAQ-based quantitative proteomic analysis reveals key pathways responsible for scurs in sheep (*Ovis aries*)

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Abstract

Scurs is a horn phenotype that exhibits as small corneous structures on the skull due to the deformed development of horn tissues. Previous genome-wide association analysis of scurs in Soay sheep showed a significant association to the polled locus, *relaxin-like receptor* 2 (*RXFP*2). However, the molecular mechanism underlying the development of scurs remains largely unknown. In the present study, we performed an iTRAQ-based quantitative proteomic analysis of horn tissues from both scurs and normal two-horned and four-horned individuals among Altay sheep to identify the differentially expressed proteins (DEPs) responsible for the scurs phenotype. In total, 232 proteins showed significant differential expression, and the most significant Gene ontology categories were the adhesion processes (biological adhesion (P=4.07×10^{-17}) and cell adhesion (P=3.7×10^{-16})), multicellular organismal process (single-multicellular organism process (P=2.06×10^{-11}) and multicellular organismal process (P=2.29×10^{-11})) and extracellular processes (extracellular matrix organization (P=4.77×10^{-16}) and extracellular structure organization (P=4.93×10^{-16})). Kyoto encyclopedia of genes and genomes (KEGG) analysis showed that extracellular matrix (ECM)-receptor interactions and focal adhesion pathways were the most significant pathways. This finding is consistent with the reduced formation of extracellular matrix in scurs and the development of deformed horn tissues. Our study helps to elucidate the inheritance pattern of sheep horn traits from the perspectives of downstream expressed proteins.

Keywords: scurs, sheep, iTRAQ, horn development, ECM-receptor interaction pathway

1. Introduction

Horns are not only closely related to natural and sex selection (Johnston *et al*. 2013), but also exhibit a variety of phenotypes, including normal two horns, scurs (deformed horns) (Ibsen 1944), polled (absence of horns) (Ryder 1983) and multi-horns (more than two horns) (Elwes 1913; Ritchie 1913; Blunn 1943; Maiwashe and Blackburn 2004). White and Ibsen (1936) originally proposed a horn inheritance

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model regarding the polled and scurs phenotypes. Recent, the *Ho* locus was identified to be responsible for the polled phenotype and mapped to *relaxin-like receptor* 2 (*RXFP*2) on chromosome 10 in domestic sheep (Smith *et al*. 1985; Chiquetehrismann 1995; Dominik *et al*. 2012). Horn size and length have also been mapped to the same *RXFP*2 locus in Soay sheep (Imanakayoshida *et al*. 2001; Johnston *et al*. 2011; Miao *et al*. 2016). The polycerate locus that encodes the multi-horned phenotype, a rare and ancient trait, was located at the genomic region OAR2: 131.990–133.525 Mb. These findings suggested that the polycerate locus and polled locus are two distinct genes in the sheep genome (Greyvenstein *et al*. 2016; He *et al*. 2016; Kijas *et al*. 2016; Ren *et al*. 2016).

Scurs are small and irregular corneous structures that develop in the same area as normal horns but are not firmly attached to the skull (Asai *et al*. 2004; Johnston *et al*. 2011; Tetens *et al*. 2015). As an unfavorable trait for production of animal husbandry, scurs exist in many sheep breeds (Wang *et al*. 2014) and reduce the value of an animal (Asai *et al*. 2004). The scurs phenotype has existed in domestic animals since 3800–3500 BC (Kyselý 2010). A previous study indicated that the Sc locus was responsible for the scurs phenotype (White and Ibsen 1936). Subsequent studies mapped the scurs phenotype to the *RXFP*2 gene on chromosome 10 in Soay sheep and wild bighorn sheep (Johnston *et al*. 2011). Interestingly, the scurs locus coincided with the locus of the polled phenotype as well as horn size. The scurs trait in cattle was mapped to chromosomes 4 and 19 (Asai *et al*. 2004; Capitan *et al*. 2009, 2011; Mariasegaram *et al*. 2010; Allais-Bonnet *et al*. 2013; Tetens *et al*. 2015), which contains different sets of candidate genes (Georges *et al*. 1993; Brenneman *et al*. 1996; Harlizius *et al*. 1997). However, the molecular mechanism underlying the development of scurs remains largely unknown.

We have constructed a multi-horned sheep pedigree using Altay sheep since 2006, and this sheep population displays rich variation in horn phenotypes, including normal horns (two horns and multi horns), scurs (deformed) and polled phenotype (He *et al*. 2016). Therefore, Altay multi-horned sheep provide an ideal animal model to investigate the scurs phenotype in sheep. In the current study, we used iTRAQ-based quantitative proteomic analysis to screen the differentially expressed proteins (DEPs) among three groups of animals with scurs (sheep carried more than two horns which were all scurs, S+M), two normal horns (sheep carried two horns with normal development, H) and normal multi-horns (sheep carried more than two horns with normal development, M) and uncover the critical proteins that play important roles in horn development. This study provides new insights into the molecular mechanisms of scurs development.

2. Material and method

2.1. Ethics statement

All experiments were performed in accordance with relevant guidelines and regulations issued by the Ministry of Agriculture of the People's Republic of China. All experimental protocols were approved by Institute of Animal Science, Chinese Academy of Agricultural Sciences, where the experiment was conducted.

2.2. Sample collections

Eight sheep at two years of age of the three groups were investigated in the current study: the S+M group included three males with scurs and four horns, the M group included three four-horned males and the H group included two two-horned males (Fig. 1). All animals were selected from the Altay multi-horned sheep family that was established in 2006 in the sheep conservation farm of the Institute of Animal Science, Chinese Academy of Agricultural Sciences and were kept under identical conditions. The horn samples were collected at the junction of horn sheath and horn marrow, and were frozen in liquid nitrogen immediately for iTRAQ and Western blot analyses.

2.3. Protein extraction, digestion and labeling

Approximately 0.5 g each of horn marrow and sheath sample were ground into powder with liquid nitrogen

Fig. 1 Altay sheep with two horns which are normal development (A), four horns which are normal development (B), five horns which are normal development (C) and four horns which are scurs (D).

separately and added to 10 mL cooled acetone containing 10% trichloroacetic acid (TCA; w/v) for protein extraction. After being centrifuged at 15 000×g for 15 min at 4°C, the precipitate was washed with cooled acetone and dried in a vacuum freeze dryer. The precipitate was subsequently dissolved in lysis solution containing 1 mmol L^{-1} phenylmethanesulfonyl fluoride (PMSF) for 1 h in a water bath at 30°C. The resulting solution was centrifuged at 15 000×g for 15 min at room temperature. The supernatant was collected and centrifuged again to obtain the supernatant containing the extracted protein (Damerval *et al*. 1986). Subsequently, protein concentrations from each sample were measured by the bicinchoninic acid (BCA) method (Smith *et al*. 1985). In each individual, the same amount of proteins from the horny marrow and sheath were pooled together at a 1:1 weight ratio.

After reduction and alkylation, 100 μg protein for each sample was digested by Trypsin Gold (Promega, Madison, Wisconsin, WI, USA) at a 40:1 ratio (2.5 μg trypsin:100 μg target) and incubated at 37°C for 12 h, and the peptides were subsequently collected. The peptides were labeled with the 8 plex iTRAQ reagent according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Peptides from the M group were labeled with iTRAQ tags 113, 114 and 115, the S+M group with iTRAQ tags 116, 117, and 118 and the H group with iTRAQ tags 119 and 121. All labeled peptides were incubated at room temperature for 2 h.

2.4. 2D-LC-MS/MS analysis

Dry samples were re-suspended in 100 μL buffer A (25 mmol L^{-1} NaH_2PO_4 in 25% ACN, pH 2.7) and SCX (strong cation exchange) was performed on an Agilent 1200 HPLC System (Agilent). Separation was performed at a flow rate of 0.3 mL min^{-1} using a nonlinear binary gradient starting with buffer A and subsequently transferring to buffer B. The first segment was collected for 5 min, and the following segments were collected ten times, each time collected for 4 min. The last segment was collected for 5 min. A total of 12 fractions were dried in a vacuum freeze dryer for LC-MSMS analysis. Samples were re-suspended in Nano-RPLC buffer A (0.1% FA, 2% ACN) and were analyzed in an online Nano-RPLC combined with an Eksigent NanoLC-Ultra™ 2D System (ABI, USA). Data acquisition was performed with a Nanospray® III source (ABI, USA) on a Triple TOF 5600 System (ABI, USA) (Andrews *et al*. 2011).

2.5. Database search and analysis

The MS/MS data were searched against the database Uniprot_Ovis aries_9940 (27 271 sequences). Data analysis was performed with Protein Pilot Software v. 5.0 (ABI, USA) using the Paragon algorithm. The false discovery rate (FDR) was calculated as the false positive matches divided by the total matches. The identified proteins were accepted within a 95% confidence interval and the threshold of unused score was set to 1.3. FDR for peptide and protein identification was set to 1%, and at least one unique peptide was required for protein identification. All samples were compared with each other, and proteins with average fold changes in abundance higher than 1.5 or lower than 0.67 were considered to be DEPs.

2.6. Bioinformatics analysis of DEPs

The Venn diagram based on DEPs was created using Venny2.1 software (http://bioinfogp.cnb.csic.es/tools/venny/). Gene ontology (GO) annotations and Kyoto encyclopedia of genes and genomes (KEGG) classifications were performed by a multi-omics data analysis tool, OmicsBean software (http://www.omicsbean.com:88/). The criteria of gene enrichment fold less than 3 and *P*-value smaller than 0.05 were used to identify the significantly enriched GO terms and KEGG signal pathways among DEPs.

2.7. Western blot verification

According to protein molecular mass, 10% separating gels and 5% stacking gels were chosen to separate proteins. The proteins were subsequently transferred onto 0.45-μm polyvinylidene fluoride (PVDF) membranes (GE Healthcare, UK) and blocked in the 5% nonfat milk (Millipore, USA) for 1 h at room temperature. The primary antibodies for Western blot analysis contained ACAN mouse monoclonal antibody (1:100, Abcam, Cambridge, UK) and beta-actin rabbit polyclonal antibody (1:5000, Abcam, Cambridge, UK). Goat anti-rabbit IgG and goat anti-mouse IgG were labeled with Horseradish Peroxidase (HRP) as secondary antibodies. The membrane was rinsed 3 times with 5% TBST for 10 min each time. The proteins were detected by means of the ECL Chemiluminescence Reagent Kit (Millipore, USA) and imaged using the Image Quant LAS 4000 Mini System (GE Healthcare, UK).

3. Results

3.1. Protein profile by iTRAQ analysis

In this study, we performed quantitative proteomic analysis based on iTRAQ and LC-MS/MS technology to identify the essential proteins in the horn development by screening the proteins that have differential expression in scurs, and horn tissues with normal development (two-horned

and multi-horned). According to the standards of protein identification, a total of 2 356 proteins were identified from 17 833 peptides by searching against the Uniprot_Ovis aries database. Among them, 1 345 proteins were identified with an FDR less than 1%.

We identified a total of 232 DEPs with average fold changes <0.67 or >1.5. Among these DEPs, 98 occurred in the comparison between M and S+M groups with 29 being down-regulated and 69 up-regulated (Appendix A). We found 151 DEPs in the comparison between H and S+M groups, with 55 proteins being down-regulated and 96 up-regulated (Appendix B). A Venn diagram showed 54 overlapping DEPs in the two comparisons, with 15 (27.8%) down-regulated and 39 (72.2%) up-regulated (Fig. 2).

3.2. GO enrichment and KEGG pathway analysis of DEPs

We performed GO enrichment analysis for 232 DEPs. In the comparison between M group *vs.* S+M group, 98 DEPs were classified in biological processes (BP), cellular components (CC) and molecular functions (MF) ontology categories, which involved 371, 55 and 53 terms, respectively. We found that the most significant enrichments were in the adhesion processes (biological adhesion (P=4.07×10^{-17}) and cell adhesion (P=3.7×10^{-16})), extracellular processes (extracellular matrix organization (P=4.77×10^{-16}) and extracellular structure organization (P=4.93×10^{-16})) and multicellular organism process (single-multicellular organism process (P=2.06×10^{-11}) and multicellular organismal process (P=2.29×10^{-11})), with the lowest P-value being observed for biological processes (BP; Fig. 3-A). Correspondingly, most of DEPs were enriched in extracellular processes in the cellular components group, with the lowest P-value including extracellular region part, extracellular organelle, extracellular membrane-bounded organelle, extracellular region and extracellular space terms (Fig. 3-A). The molecular functions group results showed that most of DEPs were related to protein binding terms such as protein-binding, RAGE receptor binding, haptoglobin binding and protein complex binding.

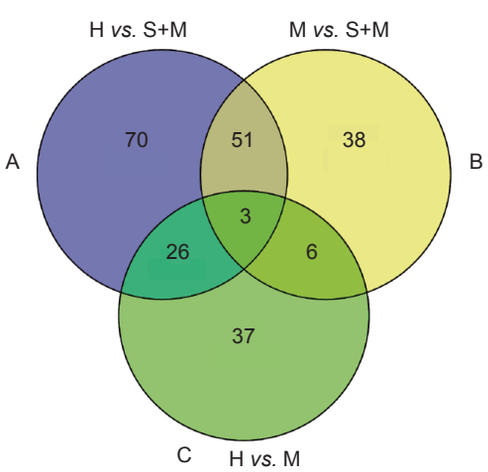

Fig. 2 Venn diagram of the three groups. A, the purple circle represents the differentially expressed proteins (DEPs) between the H (sheep carried two horns with normal development) and S+M (sheep carried more than two horns which were all scurs) groups. B, the yellow circle represents the DEPs between the M and S+M groups. C, the green circle represents the DEPs between the normal H and M (sheep carried more than two horns with normal development) groups.

In the comparison between H and S+M groups, 151 DEPs were enriched in the BP, CC and MF groups, which involved 230, 103 and 37 terms, respectively. Interestingly, 7 of 10, 8 of 10, and 4 of 10 GO terms were consistent with the GO results of the comparison between S+M and M groups (Fig. 3-B), which supports the reliability of the results.

We performed the KEGG analysis based on the DEPs among the three different groups. Twelve pathways were significantly enriched in the comparison between M and S+M group (Fig. 4-A). The top five significant pathways involved EMC-receptor interaction (hsa04512, P=9.48×10^{-9}), focal adhesion pathway (hsa04510, P=2.0×10^{-6}), protein digestion and absorption (P=4.49×10^{-4}), dilated cardiomyopathy (P=4.98×10^{-4}) and the PI3K-Akt signaling pathway (hsa04151, P=8.54×10^{-4}). The most significant pathway related to EMC-receptor interaction involved 9 DEPs, including thrombospondin 1 (THBS1), collagen type VI alpha 2 (COL6A2), collagen type VI alpha 3 (COL6A3), collagen type VI alpha 1 (COL6A1), collagen type I alpha 2 (COL1A2), integrin alpha V (ITGAV), fibronectin 1 (FN1), tenascin C (TNC) and tenascin N (TNN), and all of these were up-regulated. Among these proteins, COL6A2, COL6A3, COL6A1 and COL1A2 belonged to the collagen superfamily. Interestingly, the above nine proteins were also enriched in the other two pathways, the focal adhesion pathway and PI3K-Akt signaling pathway, which belonged to the five most significantly pathways. The EMC-receptor interaction pathway, focal adhesion pathway and PI3K-Akt signaling pathway were enriched for the largest number of 10, 9 and 10 proteins of all 12 enriched pathways. The results suggested that the three pathways may play important roles in the normal development of sheep horn, and they might have the similar biological functions.

In the comparison between H and S+M groups (Fig. 4-B), EMC-receptor interaction, focal adhesion pathway and PI3K-Akt signaling pathway were also included in the five most significant pathways. At same time, three pathways were enriched for the largest number of proteins in all 12 enriched pathways. These results supported the reliability of our study.

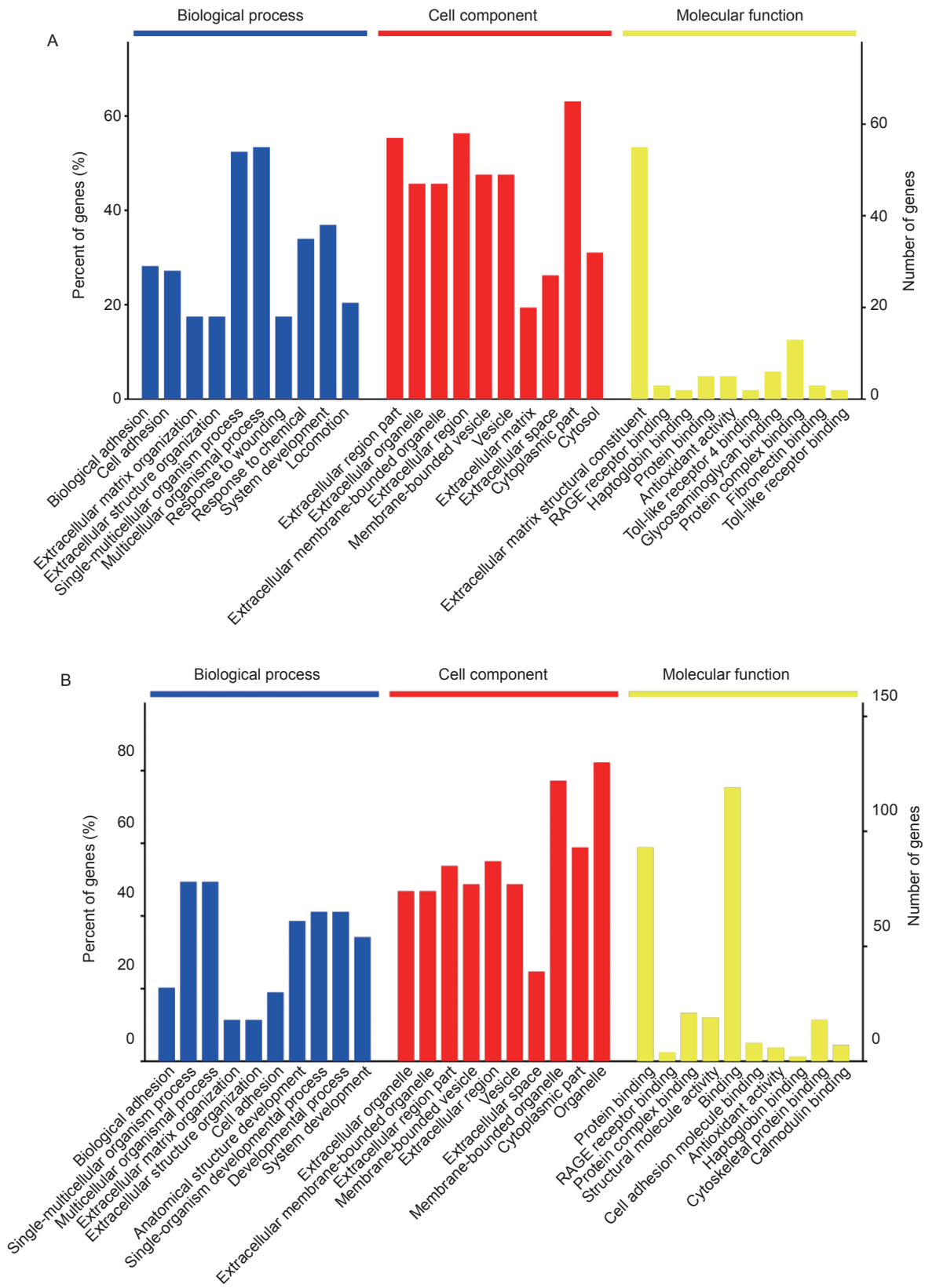

Fig. 3 Gene ontology (GO) annotations based on the differentially proteins, including biological process (BP), cellular component (CC) and molecular function (MF) between M (sheep carried more than two horns with normal development) and S+M (sheep carried more than two horns which were all scurs) groups (A) and between H (sheep carried two horns with normal development) and S+M groups (B).

3.3. Western blot validation

Because of the lack of monoclonal antibodies, and the difficulty of extracting RNA from horn tissue of adult sheep, the verification of DEPs was difficult. One of the DEPs, the aggrecan (ACAN), was selected for validation of proteomic data by Western blot. The results showed that the gray value ratio of Western blot was consistent with the ACAN ratio of three groups from iTRAQ (Fig. 5-A and B), which indicated the credibility of our proteomics data.

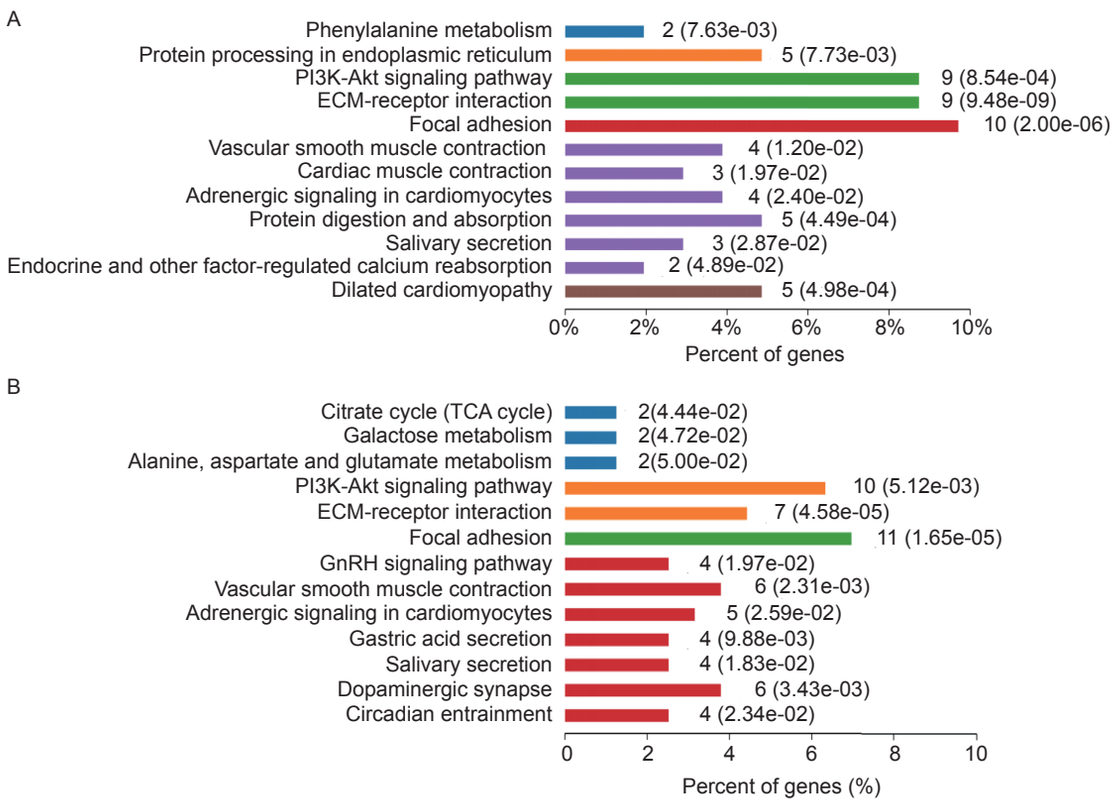

Fig. 4 Distribution of enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways in the comparison of between M (sheep carried more than two horns with normal development) and S+M (sheep carried more than two horns which were all scurs) groups (A) and between H (sheep carried two horns with normal development) and S+M groups (B). The digits represent gene numbers and *P*-values.

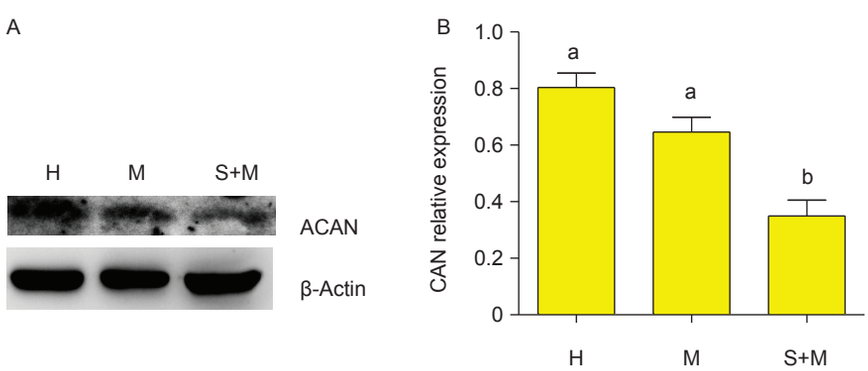

Fig. 5 Western blot verification. A, Western blot of ACAN and β-Actin as a loading control. B, ACAN quantitative analysis of the Western blot intensity. H group, sheep carried two horns with normal development; M group, sheep carried more than two horns with normal development; S+M group, sheep carried more than two horns which were all scurs. Error bars represent standard deviation. Different letters represent significant difference at $P<0.05$.

4. Discussion

Compared with previous iTRAQ results from ovary tissues (Miao *et al*. 2016), we identified fewer differentially expressed proteins (232 DEPs) from horn tissues in the current study. This may be related to the highly differentiated characteristics of horn tissues. We set up two the control groups (M and H groups) to decrease the false positive results, and we observed that 54 DEPs (55.1%) were consistent in the two pairs of comparisons: M group *vs*. S+M group and H group *vs*. S+M group. This finding demonstrated the reliability of our results. Among the 54 overlapping DEPs, we realized that more DEPs were up-regulated proteins (63.6–72.2%) in the two groups with normal horn (H and M groups), which suggested that these proteins might be associated with the normal development of sheep horn.

In the pathway analysis, the top three significant pathways were focused on ECM-receptor interaction, focal adhesion and PI3K-Akt signaling in the comparison between M and S+M groups, and these were also enriched in comparison between H and S+M groups. The results of a previous study indicated that these three pathways are involved in cell survival and cellular communication (Satoh *et al*. 2015). Both pathways of ECM-receptor interaction and focal adhesion (Myking *et al*. 2011) participate in the same cell adhesion function (Albelda and Buck 1990). Qin *et al*. (2015) showed that the PI3K-Akt signaling pathway can mediate the expression of extracellular matrix molecules in epithelial cells. In addition to regulating the focal adhesion dynamics, the focal adhesion pathway also mediates reparative bone formation *in vivo* (Castillo *et al*. 2012). Interestingly, a previous study in cattle showed that the ECM-receptor interaction pathway was the most significant pathway differentiating the scurs and polled group based on the transcript profiling (Mariasegaram *et al*. 2010). There are five proteins in this pathway, including COL1A1, COL1A2, COL5A1, COL5A2 and COL6A6 that are from the collagen protein family. The results suggested that the ECM-receptor interaction pathway may be critical for horn development (scurs) during newborn and adult stages. At the same time, the four DEPs, COL6A2, COL6A1, TNC and TNN, are overlapping proteins (Table 1) involved in the most significant pathway of ECM-receptor interaction, focal adhesion and PI3K-Akt signaling pathway in the comparisons between M and S+M groups, as well as between H and S+M groups.

A previous study showed that COL6A2 and COL6A1 belong to the collagen superfamily (Ricardblum 2011) and are important structural proteins of the extracellular matrix. The collagens deposited in the extracellular matrix not only provide structural support for organs and tissues (Hynes 2009) but also participate in cell-matrix interactions (Ricardblum 2011). The collagens also play a role as adhesion proteins (Heino 2007). TNC and TNN are types of extracellular matrix proteins and belong to the tenascin family (Chiquetehrismann 1995). They are believed to be involved in the morphogenesis of multiple organs and tissues. A previous study showed that TNC modulates the adhesion of cardiomyocytes to the extracellular matrix (Imanakayoshida *et al*. 2001). This finding implies that these four proteins may be the key proteins affecting the formation of scurs and that the main differences between deformity and normal horns exist in the extracellular matrix.

Table 1 Three most significant pathways in the comparison between H and S and between M and S groups in KEGG analysis

Group[1)]	Pathway	No. of proteins	Proteins[2)]	*P*-value[3)]
M *vs.* S+M	ECM-receptor interaction	9	THBS1, COL6A2, COL6A3, COL6A1, COL1A2, ITGAV, FN1, TNC, TNN	9.48e-09
	Focal adhesion	10	THBS1, COL6A2, COL6A3, PARVA, COL6A1, COL1A2, ITGAV, FN1, TNC, TNN	2.00e-06
	PI3K-Akt signaling pathway	9	THBS1, COL6A2, COL6A3, COL6A1, COL1A2, ITGAV, FN1, TNC, TNN	8.54e-04
H *vs.* S+M	ECM-receptor interaction	7	COL6A2, COL6A1, LAMB2, CHAD, TNC, TNN, VTN	4.58e-05
	Focal adhesion	11	ZYX, MYLK, COL6A2, PARVA, COL6A1, LAMB2, CHAD, VASP, TNC, TNN, VTN	1.65e-05
	PI3K-Akt signaling pathway	10	GNB2, COL6A2, YWHAZ, COL6A1, LAMB2, CHAD, PPP2R2B, TNC, TNN, VTN	5.12e-03

[1)] M group, sheep carried more than two horns with normal development; S+M group, sheep carried more than two horns which were all scurs; H group, sheep carried two horns with normal development.

[2)] THBS1=thrombospondin 1; COL6A2=collagen type VI alpha 2; COL6A3=collagen type VI alpha 3; COL6A1=collagen type VI alpha 1; COL1A2=collagen type I alpha 2; ITGAV=integrin alpha V; FN1=fibronectin 1; TNC=tenascin C; TNN=tenascin N; PARVA=parvin alpha; LAMB2=laminin subunit beta 2; CHAD=chondroadherin; VTN=vitronectin; ZYX=zyxin; MYLK=myosin light chain kinase; VASP=vasodilator-stimulated phosphoprotein; YWHAZ=tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta; GNB2=G protein subunit beta 2; PPP2R2B=protein phosphatase 2 regulatory subunit beta.

[3)] *P*-values were calculated based on a modified Fisher's exact test from DAVID.

5. Conclusion

In this study, a total of 232 DEPs were identified among three groups: the scurs group (S+M), the multi-horned group (M) and the two-horned group (H). GO analysis indicated that the DEPs between the normal group and the scurs group were involved in adhesion, multicellular organismal processes and extracellular structure. KEGG pathway analysis showed that ECM-receptor interaction, focal adhesion, and the PI3K-Akt signaling were the most significant pathways that may affect the formation of the extracellular matrix in horn and ultimately lead to the development of deformed horn tissue.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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