Comparative tail fat transcriptome analysis of key genes and pathways activated in response to fat deposition in two sheep breeds with extreme fat-tail phenotype differences

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Abstract

Background

Fat tail in sheep presents a valuable energy reserve that has historically facilitated adaptation to harsh environments. However, in modern intensive and semi-intensive sheep industry systems, breeds with leaner tails are preferred. For efficient selection of lean sheep breeds, clarification of the regulatory mechanisms underlying tail fat deposition of sheep is crucial. Altay and Xinjiang Fine Wool (XFW) sheep, two important breeds with distinct tail fat deposition properties, are mainly distributed in the Xinjiang district of China, and serve as ideal models for investigating the mechanisms of tail fat deposition. In the present study, RNA-Seq was applied to determine the transcriptome profiles of tail fat tissues in these two breeds, followed by analysis of differentially expressed genes (DEGs) and their sequence variations.

Results

In total, 21,527 genes were detected, among which 3,965 displayed significant expression variations in tail fat tissues of the two sheep breeds, including 707 upregulated and 3,258 downregulated genes. Gene Ontology (GO) analysis disclosed that 198 DEGs were related to fat metabolism. In Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, the majority were significantly enriched in adipocytokine signaling, PPAR signaling, and metabolic pathways, with some genes being involved in multiple pathways. Among the 198 DEGs, 22 genes were markedly up or down regulated in tail fat tissue of Altay sheep, supporting their association with the fat tail trait of this breed. A total of 41,724 and 42,193 SNPs were detected in tail fat tissue transcriptomes of Altay and XFW sheep, respectively. The distribution of 7 SNPs in the coding regions of the 22 candidate genes was further investigated in three sheep populations with distinct tail types. In particular, the g.18167532 T/C mutation of *ABCA1* and g.57036072 G/T mutation of *SLC27A2* showed significantly different distributions and were closely associated with tail type.

Conclusions

The present study provides transcriptomic evidence explaining the differences in fat- and thin-tailed sheep breeds and reveals numerous DEGs and SNPs associated with tail type. Our data provide a valuable theoretical basis for selection of lean sheep breeds.

Background

Fat tail is a valuable trait that helps sheep (*Ovis aries*) adapt to harsh conditions, such as extremely cold winters, food shortages and drought seasons. Fat-tailed sheep characteristically deposit a mass of fatty tissue in the tail region during summer and autumn seasons when nutritious pastures are available. In the winter seasons when temperatures are extremely low and grasslands are covered with heavy snow for long periods of time, these animals obtain energy by decomposing the fat deposits in their tails to sustain life. According to archaeological findings, modern sheep breeds were domesticated at the Fertile Crescent district of Iraq about 9,000 years ago [1–3]. Similar to their Asian mouflon ancestor, modern breeds were thin-tailed at the early stages. Fat-tailed sheep appeared ~ 5,000 years ago through long-term artificial and natural selection during the long evolution process to adapt to harsh local climate conditions [4–7]. However, in modern society, dietary habits and health concepts have

undergone a profound revolution, and mutton with lower fat content is preferred for consumption. Furthermore, fat deposition requires more energy than deposition of lean tissue. Thus, the efficiency of meat production is higher than that of fat in intensive and semi-intensive sheep industry systems, and the tail fat of Altay sheep (comprising up to ~ 25% of carcass weight) markedly lowers their economic value [8]. For the above reasons, fat-tailed sheep breeds have been gradually rejected by producers and consumers, and leaner sheep breeds are more desirable [9–14]. Therefore, elucidation of the key genes that regulate the deposition and decomposition of tail fat in sheep and the molecular mechanisms controlling fat metabolism should greatly accelerate the breeding course of lean sheep and production of more healthy mutton for consumption.

Fat is not only used for energy storage but also an important endocrine tissue involved in regulating crucial physiological and biochemical reactions in organisms [15–18]. Traditionally, research on the molecular regulatory mechanisms of sheep tail adipose tissue development and deposition has involved investigation of the functions of single candidate genes. Earlier, Kumar and colleagues reported higher expression of the leptin gene in perirenal, backside and omental fat tissue of fat genotype than lean genotype Coopworth sheep [19]. Both activity and expression of lipoprotein lipase (LPL) were increased in fat tissue of the fat genotype sheep groups. Moreover, its expression was tissue-specific, but not affected by the nutrition level [20]. In Guangling Large Tail sheep, the uncoupling protein 1 (*UCP1*) gene was expressed at significantly higher levels in perirenal fat than other tissues, but showed very low expression in subcutaneous fat [21]. The cell death-inducing DFFA-like effector c (*CIDEc*) gene was highly expressed in rump fat tissue of Altay sheep, which decreased significantly after a 4-week fasting period [22]. Expression of fatty acid binding protein 4 (*FABP4*) was significantly elevated in tail fat of Lori-Bakhtiari, a fat-tailed sheep breed, compared to Zel, a thin-tailed sheep breed [23]. Heart fatty acid-binding protein (*H-FABP*) gene in skeletal muscle of Lanzhou fat-tailed sheep was up or downregulated at different developmental stages and potentially related to the meat quality traits [24]. In Altay sheep, *FABP4* was abundantly expressed in intestinal and rump fat tissues and showed no significant changes upon alteration of the nutritional status, suggesting a fundamental role in adipose metabolism [25]. The collective findings highlight the involvement of several critical genes in regulation of tail fat deposition and mobilization in sheep. In general, traits of animals, including tail size, are modulated by multiple interconnected genes that form a refined regulatory network to manage complex internal and external environments. Therefore, research specifically focusing on one or several genes cannot completely understand elucidate such a network.

In recent years, due to the rapid development of next-generation sequencing and application of RNA-Seq technology, numerous genes expressed in tail fat tissue of sheep have been identified via analysis of transcriptome data in attempts to clarify the molecular network regulating adipose metabolism in fat tail of sheep. RNA-Seq has been used successfully to investigate the genes expressed in fat tissue of sheep. Wang and colleagues applied RNA sequencing technology to compare the transcriptome profiles of two sheep breeds, Kazak (fat-tailed) and Tibetan (short-tailed). Their study led to the identification of 646 differentially expressed genes between the two sheep breeds, including 280 upregulated and 366 downregulated genes. Moreover, the genes displaying the most significant fold changes, *NELL1* and *FMO3*, were highly correlated with adipose deposition in tail [26]. Guangling Large-Tailed sheep and Small-Tailed Han sheep are two typical fat-tailed breeds in China. Upon application of RNA-Seq, a total of 4131 differentially expressed genes were determined in tail fat tissues of these two breeds, with *FABP4*, *FABP5*, *ADIPOQ* and *CD36* identified as the four most highly transcribed genes [27]. Research on Small-Tailed Han and Dorset sheep revealed 602 differentially expressed genes, and GO analysis showed that several of these genes were enriched in the triglyceride biosynthetic process [28]. RNA-Seq analysis by Ma and colleagues to investigate the tail fat transcriptome of Lanzhou Fat-Tailed (long fat-tailed), Small-Tailed Han (short fat-tailed), and

Tibetan (short thin-tailed) sheep identified several differentially expressed genes (DEGs) and long non-coding RNAs (lncRNAs). GO and pathway analysis of DEGs and target genes of differentially expressed lncRNAs revealed that the majority were enriched in fatty acid metabolism and fatty acid elongation-related pathways that contribute to fat deposition [29]. Previous studies clearly indicate that the tail fat deposition ability of sheep with different tail types is a complex quantitative trait regulated by multiple genes. The molecular mechanisms underlying tail fat deposition remain to be elucidated.

Altay and Xinjiang Fine Wool (XFW) sheep with distinct tail types are both distributed in the Xinjiang district of China. Altay is one of the most popular fat-tailed breeds. The tail and rump are fused together and these animals are characterized by their ability to deposit rump fat, which accounts for ~ 25% of carcass weight on average in this breed. In contrast, XFW, a typical long thin-tailed breed of sheep, stores almost no fat tissue in its tail. These two sheep breeds with distinct tail fat characteristics thus present ideal models for investigating DEGs involved in regulation of tail fat deposition (Fig. 1). Accordingly, we focused on rump and tail fat tissue of Altay and XFW sheep as model animals. RNA-Seq technology was applied to identify DEGs and the associated signaling pathways, with the aim of highlighting candidate genes and mechanisms that play critical roles in regulating adipose deposition in tail of sheep. Genetic variations of these DEGs were further investigated. Our collective data should provide fundamental information and theoretical guidelines for efficient breeding of lean sheep.

Results

Summary of transcriptome sequencing data

Two cDNA libraries were constructed using mRNAs extracted from fat-rumped Altay sheep and thin-tailed XFW sheep, sequenced, and two sets of raw reads were obtained containing 51,943,518 and 51,770,440 raw reads, respectively. Low-quality raw reads and adapter sequences were then filtered, ultimately resulting in 46,614,192 and 46,646,110 clean reads. Approximately 84% and 81% clean reads could be mapped to the sheep reference genome *Ovis aries* v3.1. The clean reads were finally assembled into Unigenes, which were categorized to two classes, specifically, clusters and singletons. Clusters were labeled by the prefix 'CL', followed by the cluster id. A single cluster included several Unigenes with > 70% sequence similarity. Singletons were indicated by the prefix 'Unigene' (Additional File 1, Table S1). In total, 153,914 and 117,254 clusters and 78,065 and 56,293 singletons were obtained from the two sample sets, respectively. The mean lengths of clusters were 335 nt and 317 nt, while mean lengths of singletons were 696 nt and 629 nt for Altay and XFW groups, respectively (Additional File 2, Figure S1). Clusters and singletons were further analyzed and filtered, resulting in a final total of 48,894 Unigenes. Transcriptome sequencing data are summarized in Table 1.

Table 1
Transcriptome sequencing data from Altay and XFM sheep

Samples	Altay	XFM
Total raw reads	51,943,518	51,770,440
Total clean reads	46,614,192	46,646,110
Total clean nucleotides (nt)	4,661,419,200	4,664,611,000
Q20 percentage (%)	97.97	97.86
N percentage (%)	0.01	0.01
GC percentage (%)	48.41	47.15
Error rate (%)	0.01	0.01
Total mapped reads	39,155,921	37,783,349
Multiple mapped reads	1,370,457	1,435,767
Unique mapped reads	37,785,464	36,347,582
Unmapped reads	7,458,280	8,862,761
Mapping rate (%)	84	81
Total number of clusters	153,914	117,254
Total number of singletons	78,065	56,293
Total length of clusters (nt)	51,601,654	37,173,312
Total length of singletons (nt)	54,302,714	35,416,372
Mean length of clusters (nt)	335	317
Mean length of singletons (nt)	696	629
N50 length of clusters (nt)	573	519
N50 length of singletons (nt)	1114	917

Annotation and expression analysis of Unigenes

Comparison of the Unigenes obtained with known gene sequences of *Bos taurus* and *Ovis aries* revealed a total of 21,527 genes (E-value < 0.00001), which were subsequently matched to NR (RefSeq non-redundant proteins), Swiss-Prot, KEGG and COG (Cluster of Orthologous Groups of proteins) databases, leading to 57%, 53%, 61%, and 45% annotation, respectively (E-value < 0.00001). GO analysis was applied to clarify the biological functions of the above genes (Additional File 1, Table S1).

Calculation of gene coverage revealed that 65% (13,993/21,527) genes of Altay sheep and 68% (14,638/21,527) genes of XFW sheep had 90–100% coverage (Fig. 2A, B). In total, 19,878 annotated genes with FPKM > 0 were detected in the two samples. The FPKM trends of the two samples were comparable, indicating similar expression

patterns of the majority of genes in tail fat tissues of Altay and XFM sheep (Fig. 2C). The largest proportions of genes were expressed at low ($1 < \text{FPKM} < 10$) and moderate ($10 < \text{FPKM} < 100$) levels and only a small fraction expressed at high levels ($\text{FPKM} > 100$). The results indicate that high-throughput sequencing technology has an obvious advantage in detection of low-abundance genes. Further analysis revealed 94.08% (18,701/19,878) of the total genes, including 384 uniquely expressed genes, in fat rump of Altay sheep and 93.25% (18,536/19,878) of total genes, including 219 uniquely expressed genes, in thin tail of XFW sheep. Overall, we detected 18,317 common genes in tail fat tissues of the Altay and XFW sheep (Fig. 2D).

Analysis of DEGs between tail fat tissues of the two sheep breeds

In total, 8,042 differentially expressed genes (DEGs) were identified between the two sheep breeds using $\text{FDR} \leq 0.001$ and $|\text{Log}_2\text{Ratio}| \geq 1$ as filter criteria (Fig. 3A; Additional File 3, Table S2). Within this gene set, differences in levels of 3,965 DEGs in tail fat tissues of the two sheep breeds were highly significant ($\text{FDR} \leq 0.001$ and $|\text{Log}_2\text{Ratio}| \geq 2$), including 707 highly upregulated and 3,258 highly downregulated genes in fat-rumped Altay, compared to XFW thin-tailed sheep (Fig. 3B; Additional File 3, Table S2).

To further clarify the functions of DEGs in tail fat metabolism of the two sheep breeds, we identified 198 DEGs (72 upregulated and 126 downregulated) closely related to adipose tissue development, deposition and mobilization. Among these genes, levels of *ABCA1*, *PLIN1*, *SORBS1*, *ANGPTL4*, *LPIN1*, *ELOVL5*, *ACACA*, *FASN*, *CIDEA*, *FABP3*, and *SLC27A2* were significantly higher in Altay than XFW sheep. In contrast, *CYP4A11*, *FADS2*, *PTPLB*, *ACAA1*, *PPCK1*, *PMP2*, *HSL*, *CPT1A*, *C1QTNF1*, *ACADL*, and *C1QTNF9* were more highly expressed in tail fat of XFW than Altay sheep (Additional File 3, Table S2).

Based on significant differences in expression of these genes between tail fat tissues of Altay and XFW breeds and their participation in regulation of fat metabolism, we speculate that the DEGs identified play potentially important roles in influencing phenotypes of different sheep breeds. We further focused on 22 DEGs showing highly significant up- or down-regulation in tail fat tissue of Altay sheep as candidate genes.

qRT-PCR validation of RNA-seq data

To further investigate expression patterns and validate the reliability of RNA-seq results, 22 candidate genes were selected and their relative expression levels in rump and tail fat tissue of Altay and XFW sheep, respectively, assessed via qRT-PCR using non-pooled RNA samples ($n = 3$ for each breed). qRT-PCR expression patterns of these genes were consistent with RNA-Seq data (Fig. 4; Additional File 4, Figure S2), supporting the reliability of the expression profile generated with RNA-Seq.

Among the 22 candidate genes, 10 (*ABCA1*, *ACACA*, *PLIN1*, *FASN*, *FABP3*, *SORBS1*, *ANGPTL4*, *LPIN1*, *SLC27A*, and *CIDEA*) were highly upregulated in rump fat tissue of Altay relative to tail fat tissue of XFW sheep ($P\text{value} < 0.01$). In particular, expression of *ACACA*, *ABCA1* and *CIDEA* in rump fat of Altay sheep was 6.75, 5.37 and 5.86 times higher than that in tail fat of XFW sheep (Fig. 4A). Eleven other genes (*ACADL*, *C1QTNF1*, *CPT1A*, *CYP4A11*, *HSL*, *FADS2*, *PTPLB*, *ACAA1*, *PPCK1*, *PMP2*, and *C1QTNF9*) were more highly expressed in tail fat tissue of XFW than Altay sheep ($P\text{value} < 0.01$), particularly *HSL* and *CPT1A*, which were 7.45 and 8.15 times higher, respectively (Fig. 4B). In view of these findings, we hypothesize that these genes play critical roles in regulating tail fat metabolism of Altay and XFW sheep and ultimately influence tail type.

GO and KEGG analyses of DEGs between Altay and XFW sheep

GO was applied for functional analysis of the 8,042 DEGs (Additional File 3, Table S2). GO terms with Q values ≤ 0.05 were considered significantly enriched and DEGs classified based on 'cellular component', 'molecular function', and 'biological process' categories. In total, 847 terms were enriched in cellular component, of which 24 were significantly enriched, such as 'membrane', 'membrane part', and 'cell periphery' (Fig. 5A). Overall, 7,014 biological process terms were enriched, 71 to a significant extent, including 'cell communication', 'response to stimulus', and 'multicellular organismal process' (Fig. 5B). Among the 1,903 terms enriched in molecular function, 32 were significantly enriched, including 'substrate-specific transporter activity', 'insulin receptor binding', and 'protein kinase A regulatory subunit binding' (Fig. 5C).

To identify the biological pathways involved in fat deposition, DEGs were mapped to the KEGG pathway database. Pathways with Q values ≤ 0.05 were considered significantly enriched (Additional File 3, Table S2). In total, 256 pathways were enriched, among which 134 were significantly enriched. The top 20 pathways, including 'MAPK signaling', 'Insulin signaling', 'Jak-STAT signaling', and 'Phosphatidylinositol signaling', are listed in Fig. 5D. We identified 245 and 187 DEGs enriched in the MAPK and insulin signaling pathways, respectively, indicating key roles of these mechanisms in tail/rump fat metabolism of Altay and XFW sheep.

GO and KEGG analyses of DEGs related to adipose metabolism

GO analysis of the 198 DEGs disclosed significant enrichment of the molecular function terms 'catalytic activity', 'molecular function', and 'transferase activity'. With regard to biological process terms, 'lipid metabolic process', 'small molecule metabolic process', 'oxidation-reduction process', and 'single-organism metabolic process' were significantly enriched. Among cellular component terms, 'cytoplasm', 'cytoplasmic part', and 'intracellular part' were significantly enriched (Fig. 6A, B). The top 30 GO enrichment terms of DEGs are presented in Figs. 6C and D.

To identify the biological pathways underlying adipose deposition, the 198 DEGs were mapped to the KEGG pathway database. The pathways with Q values ≤ 0.05 were considered significantly enriched. Several pathways related to lipid metabolism were identified (Table 2).

Table 2
Lipid metabolism-related DEG-enriched signaling pathways

Pathway	DEGs	
	Up	Down
Ether lipid metabolism	<i>AGPS, PPAP2, SPLA2, CEPT1, PLD1, PLD2</i>	<i>ENPP2, PAFAH2, EPT1, PLA2G12A, PAFAH1B2, PAFAH1B1, MAPKBP1</i>
Sphingolipid metabolism	<i>GLA, SPTLC2, SPTLC3, SGMS, PPAP2, GLB1, KDSR, SGMS2, UGCG, SGPL1</i>	<i>CERK, ACER2, SGPP1, GALC, SPT, SPTLC1, ASAH1, SPHK1, DPL1, LACZASAH1, CER, ACER12, B4GALT6</i>
alpha-Linolenic acid metabolism	<i>ACOX1, BDHAB, CYP1A2, COX1, SPLA2</i>	<i>PLA2G12A, FADS2, FADA, FADIA</i>
Linoleic acid metabolism	<i>PTGS1, BDHAB</i>	<i>PLA2G12A, MAPKBP1, CYP2J2, CYP2C40, CYP2E1</i>
Arachidonic acid metabolism	<i>PTGS2, ATS2, EPHX2, COX1, CBR3, CBR1</i>	<i>CYP2U1, PLA2G12A, CYP4F3, GGT5, LTC4S, PTGES, CYP4A11, K15717, ALDH3A2</i>
Glycerolipid metabolism	<i>LPIN1, PLSC, LCLAT1, ALDH9A1, PPAP, GPAT12, SHROOM4, GLA, DGAT2</i>	<i>DGKA, DGKD, ALDH3A2, DGKH, AGPAT1, DGKQ, MOGAT3, GPAT4, GPAT3, ADH, LIP, ATS2, ACT2, DGAT1</i>
Biosynthesis of unsaturated fatty acids	<i>ACNAT, ELOVL5, ACOX1</i>	<i>ACOT7, FADS2, TECR, ACAA1, BAAT, RPB1, PTPLB</i>
Steroid hormone biosynthesis	<i>PGFS, UGT2B7</i>	<i>CYP7B1, CYP1B1, CYP1A1, AKR1C2, AKR1C4, AKR1C, HSD11B1, HSD11B2,</i>
Steroid biosynthesis	<i>SOAT1, ACNAT2, ACOT8, PODNL1, TM7SF2</i>	<i>CYP2R1, SC5DL, AKR1C4, HSD17B4, SCP2, ERG24, LBR, ERG3</i>
MAPK signaling pathway	<i>AKT2, AKT3</i>	<i>PLA2G12A, TNFRSF1A, CHUK, IKBKB, MAPK8</i>
Fatty acid degradation	<i>FADA, HELZ, ADIPOQ, C5ORF25, MFSD4, CPT, ACOX1, FADD, ACOX3, ACSL1, ACSL3, ACSL4, ACSL6</i>	<i>CPT1A, ALDH3A2, ACADL, ACAT1, HADHB, ECHS1, CYP4A11, ACAA1, PAAF, ECHDC3, HADHA</i>
Fatty acid elongation	<i>ELOVL5, C5ORF25</i>	<i>HADHB, ECHS1, ACAA2, SMR3A, HELZ, PTPLB, HACD, TECR, ACOT7</i>
Fatty acid biosynthesis	<i>FASN, ACACA, CBR4</i>	<i>TNS, TENC1</i>
Fat digestion and absorption	<i>ABCA1, DGAT2, AGPAT1, LPPR3</i>	<i>CD36, APOA1, PLA2G12A, SCARB1, MOGAT3, MAPKBP1, GOT2, CALB2, LPAAT</i>
Adipocytokine signaling pathway	<i>AKT2, AKT3, ACSL1, ACSL3, ACSL4, ACSL6, ACACA, ACACB, CD36, SLC27A2, FADD, G6PC, RXRB, OPTN,</i>	<i>MAPK8, PRKAB1, PRKAB2, IRS2, CPT1A, ADIPOR2, ADIPOQ, PPARA, PPCK1, C1QTNF9, RXRA, PTPN11, LEP, CAMKK2, JAK2, STAT3, TNFRSF1A, TNFRSF1B, CHUK, IKBKB, MTOR, SLC2A4, NFKBIA, NFKBIB, ACSBG2, PCK2, PEPCK, STK11, C1QTNF1, PRKAG2</i>

Pathway	DEGs	
	Up	Down
PPAR signaling pathway	<i>FABP3, FABP4, SLC27A2, SLC27A6, ACSL1, ACSL3, ACSL4, ACSL6, PLIN1, ANGPTL4, SORBS1, PDPK</i>	<i>ADIPOQ, ACADL, AQP7, APOA1, SCP2, APM-1, ACAA1, CPT1A, ACOX1, ACOX2, C1QTNF1, CYP4A11, FADS2, PPCK1, PMP2, RXRA, RXRB, DBI, C1QTNF9, TRIM56</i>
Insulin signaling pathway	<i>ACACA, SORBS1, FASN, AKT2, AKT3, G6PC, PRKAA1, PRKAB2</i>	<i>MAPK8, PPCK1, IRS4, IKBKB, PDPK1, PRKAG2, SLC2A4, MTOR</i>
Metabolic pathways	<i>FASN, LPIN1, CDS2, PLD1, AGPS, PLD2, UGCG, SGMS2, AGPAT9, G6PC, DGAT2, GPAM, SGPL1, AGPAT6, TM7SF2, CBR3, GLB1, DGKE, SPTLC2, SPTLC3, ACOX1, PTGS2, EPT1, ETNK1, ACACA, ACSL1, ACSL3, ACSL4, ACSL6</i>	<i>PGS1, ACADL, IRS2, CYP2U1, PPCK1, ALDH3A2, PAFAH2, ACAT1, CYP2R1, HADHB, PLA2G12A, PAFAH1B2, CYP4F3, PCYT1A, HSD11B1, KDSR, SC5DL, PAFAH1B1, BDH1, AGPAT1, GGT5, DGKQ, LTC4S, ASAH1, AKR1A1, ECHS1, HSD11B2, CYP1A1, UGT2B, SPHK1, CYP4A11, ACAA1, DGKA, DGKD, ACER2, GALC, DGKH, SPTLC1, PTGES</i>
Glycerophospholipid metabolism	<i>EPT1, CDS2, PLD1, GPD1L, AGPAT9, PLD2, GPD2, AGPAT6, ETNK1, LCLAT1, GPAM, DGKE</i>	<i>PGS1, DGKA, DGKD, PLA2G12A, LPGAT1, PCYT1A, LYPLA1, PCYT2, GPD1, DGKH, DGKQ, AGPAT1</i>

In total, 32 DEGs were enriched in the PPAR signaling pathway, including 12 upregulated and 20 downregulated genes. Among these, 10 (*FABP3, FABP4, SLC27A2, SLC27A6, ACSL1, ACSL3, ACSL4, ACSL6, ANGPTL4*, and *PLIN1*) were highly expressed and 16 (*ADIPOQ, ACAA1, ACADL, AQP7, APOA1, SCP2, APM-1, CPT1A, ACOX1, ACOX2, C1QTNF1, CYP4A11, FADS2, PPCK1, RXRA*, and *PMP2*) were significantly downregulated in rump fat of Altay sheep.

Furthermore, 44 DEGs were enriched in the adipocytokine signaling pathway, including 14 upregulated and 30 downregulated genes. Ten of these genes (*AKT2, AKT3, ACSL1, ACSL3, ACSL4, ACSL6, ACACA, ACACB, CD36*, and *SLC27A*) were significantly upregulated and 11 genes (*MAPK8, PRKAB1, PRKAB2, IRS2, CPT1A, ADIPOR2, ADIPOQ, PPARG, PPCK1, C1QTNF9*, and *RXRA*) were significantly downregulated in rump fat of Altay sheep.

Other pathways, including 'Metabolic pathway', 'Insulin signaling pathway', 'Glycerolipid metabolism', additionally play important roles in fat metabolism. The top 30 enriched pathways are presented in Fig. 7A and C. Heatmaps were generated that clearly depicted significantly enriched pathways and DEGs (Fig. 7B, D)

Since the majority of the 198 DEGs were enriched in key pathways significantly related to fat metabolism, we propose that these genes are critical for sheep tail phenotype regulation and should be further investigated.

Interaction network analysis of proteins encoded by DEGs related to adipose metabolism

With the aid of STRING and Cytoscape software, an interaction network of proteins encoded by the 198 DEGs related to adipose metabolism was constructed (Fig. 8), resulting in the detection of existing interactions among 148 genes. KEGG data disclosed 94, 37, 31, 26, 21, 25, and 19 interacting proteins related to the terms 'Metabolic pathway' ($\text{FDR} \leq 2.67\text{e-}61$), 'Adipocytokine signaling pathway' ($\text{FDR} \leq 1.07\text{e-}47$), 'PPAR signaling pathway' ($\text{FDR} \leq 9.24\text{e-}38$), 'Glycerophospholipid metabolism' ($\text{FDR} \leq 5.14\text{e-}27$), 'Fatty acid metabolism' ($\text{FDR} \leq 2.17\text{e-}25$), 'Insulin resistance' ($\text{FDR} \leq 9.38\text{e-}25$), and 'Fatty acid degradation' ($\text{FDR} \leq 1.43\text{e-}23$), respectively. In view of the important

roles of these pathways in adipose metabolism, we speculated that they may also influence the tail types of different sheep breeds by regulating fat metabolism in tail tissue.

The interaction network of 22 proteins related to fat deposition was further analyzed, which revealed interactions among 19 of the proteins (Fig. 9A). The core nodes were identified as ACOX1, FASN, and ACAA1. ACOX1 interacted with ACADS, SLC27A2, CPT1A, FASN, and ACAA1. Interactions of FASN with CPT1A, ACACB, ACACA, ACLY, and ACOX1 were detected. ACAA1 showed interactions with PEX7, HADH, ACADS, ACOX1, and ACLY. GO analysis revealed that the majority of these proteins were related to the PPAR signaling pathway, Fatty acid metabolism, and Fatty acid biosynthetic process. The expression patterns of these 22 proteins in tail/rump fat of Altay and XFWs detected via qRT-PCR and RNA-seq support their critical roles in tail type regulation of sheep through formation of regulatory feedback loops, ultimately leading to a complex network.

To further clarify the functions of *ABCA1* and *SLC27A2* showing significant SNP differences in sheep populations with different tail types, their interaction networks with other proteins were analyzed (Fig. 9B). ABCA1 showed interactions with 10 proteins, including APOA1, UGP2, ARHGEF12, AOX1 and ARHGEF11 while SLC27A2 interacted with 8 proteins, including ABCD1, AGPAT1, and HSD17B7. Based on their identification as DEGs of Altay and XFW sheep and significant association of mutations with tail type of sheep, we hypothesize that these gene variations potentially affect expression of ABCA1 and SLC27A2 and thus the functions of interacting proteins, leading to alterations in metabolism of tail fat that ultimately influence the tail type in sheep breeds.

Detection of variants in candidate genes related to tail-fat metabolism

Using GATK software package and SOAPsnp, a total of 41,724 and 42,193 SNPs were detected in tail fat tissue transcriptomes of Altay and XFW sheep, respectively (Additional File 5, Table S3; Additional File 6, Table S4). We specifically focused on the 22 candidate genes related to tail fat metabolism, which led to the identification of 13 SNPs in the coding regions of 9 genes, among which 12 induced amino acid alterations (Table 3).

Table 3
The SNPs in candidate genes related to tail fat metabolism

Gene	Position	Basic	FR-chr base	FR-chr reads	TT-chr base	TT-chr reads	Style of amino acid mutation	chromosome
<i>ACACA</i>	13081041	T	T	255	C;T	216;39	Glu-Lys	11
	13028657	A	G	255	A;G	226;28	Leu-Pro	11
<i>PPCK1</i>	57902435	C	C	94	TC	235;20	Glu-Lys	13
<i>ABCA1</i>	18100859	G	T;G	27;16	G	4	Pro-Leu	2
	18167532	G	G	166	A;G	48;14	Lys-Glu	2
<i>SLC27A2</i>	57036072	C	C	2	A;C	13;5	Met-Ile	7
<i>CPT1A</i>	45468209	T	T;G	13;11	G	4	Ser-Arg	21
	45468249	G	A;G	13;12	G	1	Ser-Ser	21
<i>FBP2</i>	31747535	G	A	4	G;A	27;7	Ile-Val	2
<i>FADS2</i>	31762518	C	C	2	T;C	37;16	Arg-Gly	21
	39768783	C	C	11	T;C	29;15	Arg-Trp	21
	39774594	A	G	20	G;A	35;31	Arg-Gly	21
<i>PLIN1</i>	20197576	C	C;G	251;2	T;C	189;64	Ala-Tyr	18
Note: FR, fat rump; TT, thin tail								

Using PCR-RFLP and PCR-SSCP, the distribution of seven SNPs that induced amino acid substitutions was further investigated in Altay, XFW, and Hu sheep populations with different tail phenotype (Table 4).

Table 4
Distribution of 7 SNPs in three different sheep breed populations

Gene SNP	Sheep breed	Genotype frequencies			Allele frequencies		Ratio	χ^2
<i>ABCA1</i>		AA	AG	GG	A	G	A/G	
18100859	Altay sheep (104)	0.327(34)	0.481(50)	0.192(20)	0.567(118)	0.433(90)	1.311	0.045
	XFW sheep (104)	0.212(22)	0.423(44)	0.365(38)	0.423(88)	0.577(120)	0.733	1.849
	Hu sheep (104)	0.135(14)	0.365(38)	0.500(52)	0.308(66)	0.683(142)	0.464	2.552
<i>ABCA1</i>		TT	TC	CC	T	C	T/C	
18167532	Altay sheep (104)	0(0)	0.106(11)	0.894(93)	0.053(11)	0.947(197)	0.056	0.324
	XFW sheep (104)	0.865(90)	0.096(10)	0.038(4)	0.913(190)	0.087(18)	10.556	15.97**
	Hu sheep (104)	0(0)	0.962(100)	0.038(4)	0.481(100)	0.519(108)	0.926	89.16**
<i>CPT1A</i>		GG	GT	TT	G	T	G/T	
45468209	Altay sheep (104)	0.346(36)	0.654(68)	0(0)	0.673(140)	0.327(68)	2.058	24.54**
	XFW sheep (104)	0.385(40)	0.577(60)	0.038(4)	0.673(140)	0.327(68)	2.058	10.05**
	Hu sheep (104)	0.173(18)	0.644(67)	0.183(19)	0.495(103)	0.505(105)	0.981	8.661*
<i>FADS2</i>		CC	CT	TT	C	T	C/T	
39768783	Altay sheep (104)	0.106(11)	0.894(93)	0(0)	0.553(115)	0.447(93)	1.237	68.01**
	XFW sheep (104)	0.385(40)	0.615(64)	0(0)	0.692(144)	0.308(64)	2.250	20.54**
	Hu sheep (104)	0.683(71)	0.317(33)	0(0)	0.803(175)	0.139(33)	5.303	3.698

* $P < 0.05$ ($\chi^2_{0.05} = 5.99$), ** $P < 0.01$ ($\chi^2_{0.01} = 9.21$).

Gene SNP	Sheep breed	Genotype frequencies			Allele frequencies		Ratio	χ^2
<i>FBP2</i>		AA	AG	GG	A	G	A/G	
31747535	Altay sheep (104)	0.529(55)	0.385(40)	0.087(9)	0.721(150)	0.279(58)	2.584	0.198
	XFW sheep (104)	0.644(67)	0.269(28)	0.087(9)	0.779(162)	0.221(46)	3.522	4.964
	Hu sheep (104)	0.596(62)	0.337(35)	0.067(7)	0.764(159)	0.236(49)	3.244	0.447
<i>PLIN1</i>		CC	CG	GG	C	G	C/G	
20197576	Altay sheep (78)	0(0)	0.231(24)	0.770(80)	0.115(24)	0.885(184)	0.130	1.327
	XFW sheep (78)	0(0)	0.077(8)	0.923(96)	0.038(8)	0.962(200)	0.040	0.125
	Hu sheep (78)	0(0)	0.295(30)	0.705(74)	0.147(30)	0.853(178)	0.168	2.333
<i>SLC27A2</i>		GG	GT	TT	G	T	G/T	
57036072	Altay sheep (104)	0.337(35)	0.587(61)	0.077(8)	0.630(131)	0.370(77)	1.701	6.915*
	XFW sheep (104)	0.038(4)	0.125(13)	0.837(87)	0.101(21)	0.899(187)	0.112	89.163**
	Hu sheep (104)	0.385(40)	0.596(62)	0.019(2)	0.683(142)	0.317(66)	2.151	14.70**
* $P < 0.05$ ($\chi^2_{0.05} = 5.99$), ** $P < 0.01$ ($\chi^2_{0.01} = 9.21$).								

Based on data obtained from 104 individuals of each sheep breed, the distribution of g. 18167532 T/C mutation of *ABCA1* and g. 57036072 G/T mutation of *SLC27A2* in these three populations showed significant differences. For the g. 18167532 T/C mutation of *ABCA1*, 89.4% individuals in the fat-rumped Altay sheep population were CC genotype, 96.2% of fat-tailed Hu sheep (with fat-tailed phenotype between Altay and XFW sheep) were TC genotype, and 86.5% individuals in the long thin-tailed XFW group were TT genotype. The results of the Chi-square test showed that this SNP was not in Hardy-Weinberg equilibrium in XFW and Hu sheep populations ($P < 0.01$) while the Altay population was in Hardy-Weinberg equilibrium at this site ($P > 0.05$).

For the g. 57036072 G/T mutation of *SLC27A2*, G allele was main genotype in fat-rump Altay and short fat-tailed Hu sheep populations (63.0% and 68.3% of individuals had the G allele, respectively) while in the thin-tailed XFW sheep

population, 89.9% of individuals had the T allele. The three sheep populations were not in Hardy-Weinberg equilibrium at this SNP.

Discussion

Transcriptome studies of tail/rump fat from sheep

Tail/rump fat deposition in sheep breeds distributed among high-latitude regions facilitates their adaptation to harsh environments. However, these fat-tailed/rumped sheep are undesirable for the modern sheep industry and consumers. Determination of the mechanisms underlying tail fat deposition should aid in not only improving the meat quality of existing breeds but also selecting the leaner breeds. Obesity is one of the predominant health problems in human society and fat-tailed sheep provide an ideal model to investigate fat deposition mechanisms for identifying effective approaches to solve this issue. Meanwhile, developments in RNA-Seq, a powerful transcriptome profiling technology, have facilitated elucidation of the regulatory mechanisms underlying single traits.

Transcriptomes and DEGs of tail fat tissue of sheep breeds with different tail types have been investigated in recent years [26-29], leading to the identification of several important genes and pathways related to fat deposition. However, since the sheep breeds selected for these studies have not always been suitable, the results may not comprehensively reflect differences in the molecular mechanisms regulating tail fat deposition. Guangling Large-Tailed, Lanzhou Fat-Tailed and Small-Tailed Han sheep studied by Li and Ma [27, 29] are all fat-tailed breeds that display some differences in tail shape. Their transcriptomes are highly similar, and thus limited DEGs have been identified among these breeds. Kazak and Tibetan sheep researched by Wang and colleagues are fat-tailed and thin-tailed breeds, respectively [26], but distributed in different districts of China with distinct climates and natural environments. The differences in their transcriptomes may therefore be affected by several other factors, which complicate the results. Furthermore, fat-tailed sheep breeds investigated in these earlier studies are distributed in temperate rural regions of China where the winter is not extreme and feed is sufficient over the whole year. Consequently, the speed of deposition and mobilization of tail fat in these breeds may be slower, compared to breeds in high-latitude areas.

Here, we used two highly suitable sheep breeds, fat-rumped Altay and thin-tailed XFW, for investigating transcriptome differences in tail fat tissue. These two breeds were selected for several reasons. Firstly, both groups of sheep are traditionally localized in the Xinjiang province of China, which avoids the potential effects of distinct environment factors on transcriptome data. Secondly, the tail types are extremely different. The tail and rump of Altay sheep are fused together, accounting for about 25% carcass weight on average while XFW sheep almost deposit no fat in their tails, suggestive of significant differences in their transcriptomes. Thirdly, the living environment of Altay sheep is characterized by long and cold winters and relatively short summer and autumn seasons. The average temperature across the whole year is about 0.7°C to 4.9°C and can drop as low as -51.5°C in the winter. To cope with the extreme climate, Altay sheep have the ability to rapidly deposit massive amounts of fat in the rump by consuming sufficient nutritious grass during the warmer seasons for rapid mobilization in extreme winter conditions to obtain energy and sustain life. Owing to this ability, Altay sheep can live outdoors, even at extreme temperatures of -30°C to -40°C. The two sheep breeds thus represent ideal models to compare transcriptome and DEG profiles that regulate tail fat deposition.

Using RNA-seq, a total of 19,878 genes were identified in tail fat tissues, among which 8,042 were differentially expressed between the two sheep breeds ($FDR \leq 0.001$ and $|\text{Log}_2\text{Ratio}| \geq 1$). Li and co-workers reported 5,395 DEGs between tail fat tissues of Guangling large-tailed and small-tailed Han sheep [27]. In addition, 646 DEGs between Kazak and Tibetan sheep were reported by Wang *et al.* [26], 390 DEGs between Lanzhou Fat-Tailed and Tibetan sheep by Ma *et al.* [29] and 602 DEGs between Small-Tailed Han and Dorset sheep by Miao *et al.* [28]. The numbers of total genes and DEGs identified in the current study were significantly higher than previously reported figures, which may be attributable to the higher suitability of our animal models.

Among the 3,965 DEGs with significantly different expression ($FDR \leq 0.001$ and $|\text{Log}_2\text{Ratio}| \geq 2$), 707 were upregulated and 3,258 downregulated in rump fat tissue of Altay sheep. The total number of upregulated genes was significantly lower than that of downregulated genes, similar to the trend observed by the groups of Wang and Miao [26, 28]. We speculate that the majority of downregulated genes play important roles in tail fat metabolism in thin-tailed sheep breeds from as early as the time of domestication. Under both human-induced artificial and natural selection, sheep breeds with the powerful ability of efficient tail fat deposition evolved via upregulation of genes promoting fat deposition. Since these types of sheep were more adaptable to harsh environments, their numbers gradually increased and new breeds developed, such as Altay, Guangling Large-Tailed, Lanzhou Fat-Tailed, and other fat-tailed sheep breeds worldwide.

DEGs in sheep tail/rump fat tissue

DEGs in tail fat tissue of Altay and XFW sheep may play key roles in determining tail phenotype differences. To examine this hypothesis, we focused on the 198 DEGs showing significant differences in expression ($FDR \leq 0.001$ and $|\text{Log}_2\text{Ratio}| \geq 2$) and closely related to fat metabolism. Among the KEGG pathways of these genes, 134 related to adipose metabolism were significantly enriched, including 'Adipocytokine signaling', 'PPAR signaling', 'Fat digestion and absorption', and 'Glycerolipid metabolism'.

Overall, 32 DEGs, including 12 upregulated and 20 downregulated genes, were enriched in the PPAR signaling pathway, and among which 10 (*FABP3*, *FABP4*, *SLC27A2*, *SLC27A6*, *ACSL1*, *ACSL3*, *ACSL4*, *ACSL6*, *ANGPTL4*, and *PLIN1*) were expressed in rump fat tissue at extremely high levels and 16 (*ADIPOQ*, *ACAA1*, *ACADL*, *AQP7*, *APOA1*, *SCP2*, *APM-1*, *CPT1A*, *ACOX1*, *ACOX2*, *C1QTNF1*, *CYP4A11*, *FADS2*, *PPCK1*, *RXRA*, and *PMP2*) were significantly downregulated. The PPAR signaling pathway regulates cellular differentiation, energy balance, and lipid metabolism [30]. PPAR exists as α , β , and γ isoforms [31], and expression of PPAR γ is necessary for adipocyte differentiation [32, 33]. Activation of PPAR γ is reported to be essential for deposition of intramuscular fat [34]. Previous research indicates that the majority of these upregulated genes are critical for fat deposition while downregulated genes are related to fat mobilization. In bovine mammary glands, mRNA abundance at 60 d postpartum of *FABP3* and *ACSL1* was 80- and 7-fold greater relative to 15 d antenatal, with peak expression of *SLC27A2* and *SLC27A6* at 240 and 15 d relative to parturition respectively, which are significantly associated with milk fat synthesis [35]. *ANGPTL4* promotes LPL protein intracellular degradation and triglyceride levels in adipocytes [36]. *PLIN1*, an Fsp27 activator, interacts with the CIDE-N domain of Fsp27 and markedly enhances lipid droplet growth by promoting lipid exchange and transfer [37]. *ADIPOQ*, an important adipocytokine, modulates glucose and fatty acid oxidation [38], and its polymorphisms are associated with adipose deposition in pig and cattle [39, 40]. *ACAA1* and *ACADL* play critical roles in beta-oxidation of fatty acids [41].

Another pathway enriched for DEGs (44 in total) was Adipocytokine signaling. Ten genes (*AKT2*, *AKT3*, *ACSL1*, *ACSL3*, *ACSL4*, *ACSL6*, *ACACA*, *ACACB*, *CD36*, and *SLC27A*) were significantly upregulated and 11 (*MAPK8*, *PRKAB1*, *PRKAB2*, *IRS2*, *CPT1A*, *ADIPOR2*, *ADIPOQ*, *PPARA*, *PPCK1*, *C1QTNF9*, and *RXRA*) significantly downregulated in rump fat tissue of Altay sheep. *Akt*, an upregulated gene, has three isoforms in mammals (designated *Akt1*, *Akt2*, and *Akt3*) that are implicated in the regulation of widely divergent cellular processes, such as metabolism, differentiation, proliferation, and apoptosis [42]. In mouse adipocytes, upon rapid activation of *Akt2*, glucose transporter 4 (GLUT4) translocates to the cell surface and glucose transportation is accelerated [43]. Most of the downregulated genes were negatively correlated with fat deposition. In adipose tissue of high fat diet-induced obese rats, *MAPK8* is significantly downregulated and apoptosis of adipocytes inhibited, which may be the main contributory factor to obesity [44]. Mutations of *PRKAB1* and *PRKAB2* are significantly associated with meat quality traits in pigs [45].

Upon further analysis of these DEGs and their pathways, we observed involvement of a number of DEGs in multiple pathways. For instance, *ACSL1*, *ACSL3*, *ACSL4*, and *ACSL6* contribute to regulation of Adipocytokine signaling, PPAR signaling, Fatty acid degradation, and Metabolic pathways. *MAPK8*, *AKT2*, and *AKT3* are involved in MAPK signaling, Adipocytokine signaling and Insulin signaling pathway while *FASN* and *ACACA* participate in regulation of Fatty acid biosynthesis, Insulin signaling and Metabolic pathways (Table 2). The mechanisms underlying fat metabolism are complex. Fat is not only a tissue used to store energy in animals but also an important endocrine tissue involved in regulating crucial physiological and biochemical reactions [15, 16]. Accordingly, fat metabolism is regulated by an elaborate network composed of numerous signaling pathways. We speculated that these genes involved in multiple pathways play bridging roles to connect these signaling mechanisms.

Among the 22 candidate genes, *ABCA1*, *ACACA*, and *CIDEA* were significantly upregulated in rump fat tissue of Altay sheep ($P < 0.01$) with 5.37, 6.75, and 5.86 times higher expression, compared to tail fat tissue of XFM sheep. *C1QTNF1* and *HSL* were significantly downregulated in rump fat tissue of Altay sheep with 0.15 and 0.13 times expression relative to tail fat tissue of XFM sheep ($P < 0.01$). *ABCA1* is a membrane transporter protein that plays an essential role in the efflux of cholesterol from peripheral tissues back to the liver for participating in lipid metabolism [46]. In sheep reared under intensive conditions and offered sufficient feed, *ACACA* in muscle was significantly upregulated and the fat deposition accelerated [47]. *CIDEA* (FSP27) located on the surfaces of lipid droplets of adipocytes could promote enlargement or fusion of lipid droplets via clustering and lipid transfer [48-50]. Meanwhile, *CIDEA* (FSP27) suppressed *HSL* located on lipid droplet surfaces and inhibited lipolysis [51]. In Altay sheep fasted for 4 weeks, the *CIDEA* level in rump fat tissue was significantly downregulated [22]. In human liver samples of individuals with obesity and diabetes mellitus, *CIDEA* was significantly up-regulated [52, 53]. The collective studies confirm essential roles of these candidate genes in fat metabolism.

In view of the significant differences in expression levels of these genes between tail fat tissue of Altay and XFW sheep, and their signaling pathways being closely related to fat metabolism, we speculated that the genes are key regulators of tail phenotype differences of sheep that require further investigation.

Relevance of gene variants and tail fat deposition of sheep

Previous research has confirmed that a number of gene variants are closely related to tail phenotypes of sheep. Using Ovine SNP50k BeadChip, Moradi and colleagues investigated gene variants in two Iranian sheep breeds with fat tail and thin tail phenotypes, respectively. The group identified several mutations that were significantly different between the fat-tailed and thin-tailed sheep breeds in three regions located on chromosomes 5, 7 and X [54]. Our

group additionally showed that polymorphisms of g.59571364, g.59912586, g.60149273, and g.59383635 loci on Chromosome X are markedly related to tail fat deposition ability of sheep breeds [55-57].

RNA-Seq offers novel opportunities for the efficient detection of transcriptome variants (SNPs and short indels) in different tissues and species [58, 59]. Compared to whole-genome sequencing, RNA-Seq offers a more cost-effective alternative for identifying variations and potentially causal mutations underlying the analyzed phenotypes [60, 61]. Using RNA-Seq, Suárez-Vega and colleagues detected 57,795 variants in the regions harboring Quantitative Trait Loci (QTL) for mild yield, protein and fat percentages in sheep, among which 21.44% were novel [62]. In the current study, we detected 41,724 and 42,193 SNPs in tail fat tissue transcriptomes of Altay and XFW sheep, respectively, using RNA-Seq. We further focused on SNPs in 22 candidate genes related to tail fat metabolism, 12 of which altered the encoded amino acid (Table 3).

The distributions of g. 18167532 T/C mutation of *ABCA1* and g. 57036072 G/T mutation of *SLC27A2* were significantly different in the three sheep breed populations with distinct tail phenotypes (Table 4). *ABCA1* encodes a key protein regulating apolipoprotein-mediated efflux of cholesterol and phospholipid from peripheral cells to high-density lipoprotein-cholesterol (HDL-C) [63, 64]. Most previous studies have focused on the association of *ABCA1* gene polymorphisms with human disease. The SNPs rs4149267, rs1800977, rs1800978 and rs2230806 of *ABCA1* are associated with HDL-C concentrations in Caucasian, Sacramento and French populations [65, 66]. A significant association was observed between the SNP rs3890182 and type 2 diabetes in patients of Han Chinese ancestry [67, 68]. A rs2230806 genetic variation was significantly related to the development and severity of coronary artery disease (CAD) in an Iranian population. Moreover, the K allele of *ABCA1* R219K polymorphism has been shown to exert a protective effect against CAD risk and is correlated with decreased severity of CAD, independently of plasma lipid levels [69, 70]. Here, we detected a g.18167532 T/C mutation of *ABCA1*. Its distribution in different sheep breeds was significantly related to tail fat deposition ability, which should be further investigated.

The significance of mutations in *SLC27A* isoforms has been established in previous reports. Wang and colleagues identified a SNP in exon 7 leading to an amino acid alteration in Large White and Meishan pig breeds, which was significantly correlated with growth and carcass traits [71]. SNPs at *SLC27A1* and *SLC27A4* were associated with saturated fatty acid and stearic acid contents in longissimus dorsi muscle of pig [72]. Two SNPs in exon 3 and 3'UTR of bovine *SLC27A1* exerted effects on milk production traits, such as milk protein and milk fat percentages, in Chinese Holstein cattle [73]. In the present investigation, the genotype of the g.57036072 G/T mutation of *SLC27A2* was distinct in three sheep breeds with different tail phenotypes and the G allele was significantly related to rump tail type in Altay sheep (Table 4).

The two mutations of *ABCA1* and *SLC27A2* identified in this study led to alterations in the encoded amino acids. In view of their significant association with tail phenotype of sheep, the issue of whether these mutations affect rump fat deposition in Altay sheep by influencing the functions of the corresponding proteins requires further investigation. Here, we confirmed relationships between limited SNPs and tail fat deposition traits of sheep. Further research is warranted to ascertain the associations of several other detected genes with tail fat metabolism.

Conclusions

In this research, we examined the differences in transcriptome profiles and sequences of tail fat tissue from Altay and XFW sheep breeds that are distributed within the same district of China but display distinct tail fat deposition traits by applying RNA-seq followed by qRT-PCR validation to confirm the reliability of our findings. DEGs were

identified and their functions evaluated via GO and KEGG analyses. The genes associated with fat metabolism were filtered out for further analysis. Based on the data, 22 candidate genes and two SNPs were identified that potentially contribute to differences in tail fat deposition abilities of sheep. Determination of the specific roles of these DEGs and candidate genes in tail fat deposition may aid in the selection of lean sheep breeds.

Methods

Tail fat collection and RNA extraction

Three healthy male Altay and XFW sheep (3–4 years of age) were randomly selected respectively from the sheep farm in the Animal Husbandry Institute of Xinjiang Academy of Agricultural and Reclamation Sciences, Shihezi, Xinjiang, China. The sheep were reared according to standard guidelines. At the morning, noon, and afternoon of each day, sheep were provided sufficient forage and clear water. Each evening, an additional 250 g corn per animal was supplied. Six months later, the rump region of Altay sheep was full and round owing to massive fat tissue deposition while no fat tissue was present in tail and rump of XFW sheep. Before sampling, the sheep were given anesthesia through an ear vein injection of 30 mg/kg body weight of pentobarbital sodium (Ningbo, Zhejiang, China), a kind of medium-efficiency barbitol hypnotics that could inhibit the uplink activation system of the brain stem reticular. Then the sheep were slaughtered, and 100 grams of tail fat tissue from each sheep were rapidly collected. Samples were immersed in liquid nitrogen for transportation and maintained at -80°C in the laboratory.

Total RNA was extracted from tail adipose tissue using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's protocol. The concentration and integrity of RNA were evaluated using the 2100 Bioanalyzer instrument (Agilent Technologies, Waldronn, Germany).

Blood sample collection and genomic DNA extraction

Three sheep breeds, Altay (fat rump), XFW (long thin-tailed) and Hu (short fat-tailed), were selected for analyses. From each breed, 104 individuals were randomly selected and 5 mL venous anticoagulation blood collected from each animal. Using the Blood DNA Extraction kit (Tiangen, Beijing, China), genomic DNA was extracted from blood samples. DNA concentration and purity were evaluated using the 2100 Bioanalyzer instrument (Agilent Technologies, Waldronn, Germany). The OD_{260}/OD_{280} value of DNA samples was ~ 1.8 . DNA was dissolved in TE (pH=8.0) and the concentration was adjusted to 200 ng/ μ L, then stored at -20°C before being used to detect single nucleotide polymorphisms (SNPs) in the sequences of candidate genes.

The cDNA library construction and sequencing

Total RNA from three Altay sheep was mixed to generate a cDNA library and similarly from three XFW sheep to generate another cDNA library. After treatment of total RNA with DNase I, poly(A) mRNA isolated using oligo (dT) magnetic beads (Invitrogen, California, USA). Isolated mRNA was cut into short fragments in fragmentation buffer and first-strand cDNA synthesized using random hexamer primers and reverse transcriptase. Subsequently, second-strand cDNA was synthesized, purified using the QiaQuick PCR extraction Kit (Qiagen, Hilden, Germany), and poly(A) fragment added to both ends. The short fragments were connected with sequencing adapters and separated on gels via electrophoresis. Suitable fragments were selected as templates for amplification to construct

cDNA libraries. Finally, the two libraries were sequenced using Illumina HiSeq 2000 at the Beijing Genomics Institute (Shenzhen, China).

Analysis of sequencing data

The adapter sequences, reads in which the percentage of low-quality bases (quality value $Q \leq 10$) was $>50\%$, and reads in which unknown bases were $>2\%$ were filtered. Clean reads were imported into FastQC (v0.101; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) using FastQ format for further quality control and sequencing quality evaluated. Next, clean reads were aligned against the sheep reference genome, *Ovis aries* v3.1, using Tophat 2.0 software (<http://ccb.jhu.edu/software/tophat/index.shtml>). The gene structure was analyzed via STAR (Spliced Transcripts Alignments to a Reference), the results assembled using String Tie (<https://ccb.jhu.edu/software/stringtie/>) and compared with known gene sequences of *Bos taurus* v3.1 and *Ovis aries* v3.1 applying GffCompare (<https://ccb.jhu.edu/software/stringtie/gffcompare.shtml>), and annotated to NR (RefSeq non-redundant proteins), Swiss-Prot and COG (Cluster of Orthologous Groups of proteins) databases.

Expression levels of genes were determined based on gene coverage and Fragments Per Kilobase Million (FPKM) values. The gene coverage value represents the ratio of the number of bases on the unigene covered by reads and unigene sequence length and FPKM represents fragments per kilobase of transcript per million reads mapped [74]. The FPKM method could be used to eliminate the influence of different gene lengths and sequencing levels on calculation of gene expression, allowing direct comparison of gene expression differences between two samples. Expression levels of genes were subsequently normalized and the ratios calculated using Cufflinks (<http://cole-trapnell-lab.github.io/cufflink/>). The cuffdiff module of cufflinks was applied to identify DEGs between two samples. The relative expression of unigenes in tail fat tissues of Altay and XFW sheep was determined according to $\log_2 \text{fat-rump-ratio} / \log_2 \text{thin-tail-ratio}$, and DEGs screened taking $\text{Log}_2 \text{Ratio} \geq 1$ or ≤ -1 and $P < 0.05$ as standard criteria. Finally, multiple hypothesis testing was applied to revise the P -value of each DEG. We filtered out DEGs for subsequent analyses by setting the false discovery rate (FDR) as ≤ 0.001 and absolute value of $\text{Log}_2 \text{Ratio} \geq 2$ as the threshold.

Single nucleotide polymorphisms (SNPs) of different transcriptomes were further detected using the Genome Analysis Toolkit (GATK), version 4.0.10, software package. Based on comparison of short sequences, SNP sites were filtered using SOAPsnp to obtain high-quality variants and the conditions set as variation detection quality ≥ 30 , site depth 10–100X, and adjacent SNPs >10 bp.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs

GO analysis was employed to annotate functions and further enrich the GO terms of DEGs using David online software and DEGs were mapped to GO terms in the database (<http://www.geneontology.org/>). Gene numbers for each term were ultimately calculated. Significantly enriched GO terms for DEGs were determined using a hypergeometric test. Next, the P -value was subjected to Bonferroni correction and threshold P -value ≤ 0.05 used to define significantly enriched GO terms for DEGs. Meanwhile, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.kegg.jp/kegg/pathway.html>) was applied for pathway enrichment analysis of DEGs. Pathways with Q values ≤ 0.05 were considered significantly enriched for DEGs.

Quantitative real-time PCR validation of RNA-seq results

Based on the results from GO, KEGG, and SNP detection of DEGs, 22 candidate genes related to tail fat deposition were selected, including 11 upregulated and 11 downregulated genes. The expression patterns of these genes in tail fat tissue of Altay and XFW sheep were examined via quantitative real-time PCR (qRT-PCR) to validate the reliability of RNA-Seq data. *b-actin* served as the internal control gene. qRT-PCR primers were designed using Oligo6.0 and synthesized by Sangon Biotech Co. Ltd (Shanghai, China). The 22 genes and their primers are listed in Table S5 (Additional File 7).

Using the PrimeScript[®] 1st Strand cDNA Synthesis Kit (Takara, Dalian, China), cDNAs for detection of DEGs involved in tail fat deposition were generated via qRT-PCR conducted on a Roche 480 instrument (Roche, Mannheim, Germany) using SYBR Green PCR Master Mix kit (QIAGEN, Germany) according to the manufacturer's instructions. The reaction mixture comprised 10 µL 2×Quanti Fast SYBR Green PCR Master Mix, 1 µL cDNA (<100 ng), 0.5 µL forward and reverse primers (10 µM) respectively and ddH₂O to a total volume of 20 µL. The following conditions were used for amplification: 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 65°C for 30 s, and 72°C for 7 min. qRT-PCR analysis was performed in triplicate for each sample and relative expression for each gene estimated with the $2^{-\Delta\Delta C_t}$ method. Data were analyzed using the Statistical Analysis System (version 6.12; SAS Institute, Inc., Cary, NC, USA) and results expressed as means $\pm$ SD. Significance of differences was analyzed using one-way ANOVA. Differences were considered significant at $P < 0.05$ and highly significant $P < 0.01$.

Analysis of variations in candidate functional genes involved in fat deposition

Based on SNPs analysis of candidate genes, 10 SNPs existing in coding regions of the 22 candidate functional genes involved in tail fat deposition were selected for analysis. Sequences containing the selected SNP sites were exported from transcriptome data and mapped onto the sheep genome (ISGC Oar_v3.1/oviAri3, August 2012) applying Blat of the UCSC Genome Browser (<http://genome.ucsc.edu/>), and subsequently 1,000 bp sequences around the SNP sites were cut out to design primers using Oligo 6.0 (Additional File 8, Table S6). The PCR fragment sizes were 200 to 300 bp and SNP sites were located near the middle of the amplified fragments. Primers were synthesized by Sangon Biotech Co. Ltd (Shanghai, China).

Genomic DNA of Altay (n = 104), Hu (n = 104) and XFW (n = 104) sheep was amplified using the above primers (Additional File 8, Table S6), and distribution of the 10 SNPs in these three breeds determined via Single-strand Conformation Polymorphism Analysis of PCR-Amplified Fragments (PCR-SSCP) or Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) technology. The PCR reaction mixture comprised 2.5 µL 10× PCR buffer, 0.5 µL DNA template (100 ng), 2 µL dNTPs (2.5 mM each), 0.5 µL forward and reverse primers (10 µM), 0.5 µL *Taq* DNA polymerase (5 U/µL) (TaKaRa, Dalian, China), with double-distilled H₂O to a total volume of 25 µL. The following conditions were used for amplification: 95°C for 5 min; 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 10 min. Amplified products were detected via 1.5% agarose gel electrophoresis and used for SSCP or RFLP analysis. The Hardy-Weinberg equilibrium of SNPs in the three groups was verified by calculating the expected frequencies and numbers and tested using the goodness-of-fit χ^2 test.

Interaction analysis of candidate proteins involved in adipose metabolism

STRING 11.0 (<https://string-db.org/>) and Cytoscape (<https://cytoscape.org/>) were applied to analyze the protein-protein interactions of candidate genes involved in adipose metabolism, and the interaction network illustrated.

Abbreviations

RNA-Seq: RNA sequencing; XFW: Xinjiang Fine Wool; DEGs: Differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SNP: Single nucleotide polymorphisms; CDS: Coding sequence; qRT-PCR: Quantitative real-time PCR; FPKM: Fragments per kilobase of transcript per million mapped fragments; FDR: False discovery rate; NR: RefSeq non-redundant proteins; COG: Cluster of Orthologous Groups of proteins; RFLP: Restriction fragment length polymorphism; SSCP: Single strand conformation polymorphism

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The raw reads data was submitted to the Short Read Archive (SRA) under the accession number SRR11586694-11586695 and BioProject accession number PRJNA627341 (<https://dataview.ncbi.nlm.nih.gov/?search=SUB7313538>).

Authors' contributions

WZ and SQG conceived the study. JJW, MSX, SYW and JQY performed sample collection and total RNA preparation. ZW, JJW and MSX performed the qRT-PCR validation and SNP detection. ZW and SQG conducted the data analysis and prepared figures and tables. ZW wrote the manuscript with the assistance of JJW and SQG. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental procedures were approved by the Biological Studies Animal Care and Use Committee, Xinjiang Production and Construction Corps, Peoples Republic of China, and the ethics committee of Xinjiang Academy of Agricultural and Reclamation Sciences. The ethics committee of Xinjiang Academy of Agricultural and Reclamation Sciences, P. R. China approved this study.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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Additional Files

Additional file 1: Table S1. All the annotated genes in the two libraries. (XLS 8818kb)

Additional file 2: Figure S1. Length distribution of contigs and unigenes. (PDF 730kb)

Additional file 3: Table S2. DEGs with the FDR value and the absolute value. (XLS 3549kb)

Additional file 4: Figure S2. Expression levels of 22 genes detected by RNA-seq. (PDF 444kb)

Additional file 5: Table S3. SNPs detected in Altay sheep. (XLS 4653kb)

Additional file 6: Table S4. SNPs detected in XFW sheep. (XLS 4694kb)

Additional file 7: Table S5. Primers used in qRT-PCR. (DOC 22kb)

Additional file 8: Table S6. Primers used in detecting genetic variation of candidate genes related to the lipid metabolism. (DOC 21kb)

Figures

Figure 1

A. Xinjiang Fine Wool sheep. B. Altay sheep. The pictures of sheep were taken by us.

Figure 2

A, B. Distribution of gene coverage in Altay and XFW sheep groups. C. Numbers of annotated genes with different expression levels against a range of FPKM values. D. Venn diagram of unique and shared genes in the tail fat tissues of Altay and XFW sheep.

Figure 3

A. Expression levels of genes detected in tail fat tissue from Altay and XFW sheep. B. Up and downregulated genes in tail fat tissue of Altay, compared with XFW sheep.

Figure 4

Levels of 11 upregulated genes (A) and 11 downregulated genes (B) in rump-fat of fat-rumped Altay relative to tail fat of] XFW sheep determined via qRT-PCR (* $P < 0.05$, ** $P < 0.01$).

Figure 5

GO and KEGG analysis of DEGs. A. Cell component-based classification of DEGs. B. Biological process-based classification of DEGs. C. Molecular function-based classification of DEGs. D. The top 20 enriched signal pathways of DEGs. The circle size represents the number of genes and the color signifies P-value.

Figure 6

GO analysis of genes involved in lipid deposition-related regulation. A. GO classification of upregulated genes (red, molecular function; green, biological process; yellow, cell component). C. GO enrichment of the top 30 lipid metabolism-related upregulated genes. The circle size signifies the number of genes involved in 'biological process', triangle size the number of genes involved in 'cell component', and square size the number of genes involved in 'molecular function'. The color signifies the P-value. B. GO classification of downregulated genes (red, molecular function; green, biological process; yellow, cell component). D. GO enrichment of top 30 lipid metabolism-related downregulated genes. The circle size represents the number of genes involved in 'biological process', triangle size the number of genes involved in 'cell component', and square size the number of genes involved in 'molecular function'. The color signifies P-value.

Figure 7

KEGG analysis of lipid deposition-related regulation genes. A. KEGG enrichment of the top 30 lipid metabolism-related upregulated genes. The circle size represents the number of genes and the color signifies the P-value. B. Heatmap of lipid metabolism-related upregulated genes. Green represents enriched genes. C. KEGG enrichment of the top 30 lipid metabolism-related downregulated genes. The circle size represents the number of genes and the color signifies the P-value. D. Heatmap of lipid metabolism-related downregulated genes. Green represents enriched genes.

Figure 9

A. Interaction analysis of 22 lipid metabolism-related candidate functional proteins. B. Protein interactions of SLC27A2 and ABCA1.

Supplementary Files

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- [Additionalfile8TableS6PrimersusedindetectingSNP.docx](#)
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- [Additionalfile5TableS3SNPsdetectedinAltaysheep.xlsx](#)
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