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RESEARCH ARTICLE

Integrating a genome-wide association and transcriptome analysis to provide molecular insights into growth rates in sheep

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Highlights

- Integrated genome-wide association study (GWAS) and transcriptome analysis identified *BRINP3* and *PENK* as key candidate genes regulating sheep growth rate.
- Differentially expressed genes are mainly enriched in fat metabolism and energy metabolism pathways associated with sheep growth.
- *BRINP3* and *PENK* polymorphisms are significantly associated with growth traits in Hu sheep, potentially regulating growth *via* feeding behavior.

Abstract

Investigating the genetic markers and key genes associated with sheep growth rate using integrated multi-omics approaches could provide valuable insights for the sheep industry. Based on the average daily gain (ADG), fast-growing (N_{case}=70) and slow-growing ($N_{control}$=70) Hu sheep were selected for a genome-wide association study (GWAS). A total of 10 Hu sheep (5 fast-growing and 5 slow-growing) and 10 Dorper sheep (5 fast-growing and 5 slow-growing) were selected for a comparative transcriptome analysis. Hub genes and tissue-specific genes (TSGs) were identified using weighted gene co-expression network analysis (WGCNA) and RNA sequencing (RNA-Seq) data from ten tissues, respectively. Ten genes were found within 50 kb distances of the significant single nucleotide polymorphisms (SNPs). Based on a comparative transcriptomic analysis, totals of 501 and 441 differentially expressed genes (DEGs) were identified in the HF *vs*. HS and DF *vs*. DS comparisons, respectively. Some important signaling pathways were found to be closely associated with fat metabolism and energy metabolism, such as “regulation of lipolysis in adipocytes”, “oxidative phosphorylation”, and “thermogenesis”. Several DEGs play crucial roles in fat deposition (such as *ADRB3*, *PDE3B*, *FABP4*, *SERPINE1*, *PLIN1*, and *FOXO6*) and muscle development (*MYL3*). Using the WGCNA analysis, 15 genes were considered to be hub genes associated with ADG. The integration of GWAS and RNA-Seq data indicated that *BRINP3* and *PENK* may further influence the growth rate by regulating feeding behavior in sheep. An association analysis of 1,071 Hu sheep populations revealed that mutations in the *BRINP3* (*BRINP3* g.16903465 T>C) and *PENK* (*PENK* g.39289926 T>C) genes were significantly related to the growth traits (P<0.05). This study provides novel insights into the molecular mechanisms underlying growth traits in sheep, and the *BRINP3* and *PENK* genes may be potential key candidate genes related to sheep growth rate.

Keywords: sheep growth rate, GWAS, transcriptomics, *BRINP3*, *PENK*

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1. Introduction

As widely distributed agricultural animals, sheep are primarily raised for meat and other agricultural products. Hu sheep is a valuable native sheep breed in China that is known for its many excellent traits, including year-round estrus, high prolificacy, good growth performance, and tender meat (Feng *et al.* 2018). As an ideal breed for improvement, the Dorper sheep is famous for its rapid growth, high meat production, and strong adaptability (Bai *et al.* 2020). The growth rate is a crucial factor that affects the profitability of the sheep production industry. Accelerating the improvement of growth-related traits can increase the supply of mutton. During production, the average daily gain (ADG) is used to reflect the growth rate of sheep, and it affects the market weight of slaughter lambs and commercial production for mutton (Mahala *et al.* 2020). Growth traits are complex quantitative traits influenced by multiple genes and their interactions (Walling *et al.* 2004). It is important for the sheep industry to investigate the mechanisms driving growth rates and further understand the functions of candidate genes.

RNA sequencing is a powerful high-throughput method used for the quantitative analysis of gene expression across the entire transcriptome. Several studies have identified numerous candidate genes and genetic variations associated with growth traits in sheep. For example, Cheng *et al.* (2020) identified 30, 116, and 32 differentially expressed genes (DEGs) related to growth, development, and meat quality in sheep (e.g., *FGFRL1*, *ITGB3*, *BMP2*, and *PPARD*) by comparative transcriptomic analysis. Wijayanti *et al.* (2022) found that an insertion within the *ETAA1* gene was significantly associated with growth traits in several Chinese sheep breeds (including Luxi Blackhead sheep, Hu sheep, Lanzhou fat-tailed sheep, and others) by correlation analysis. Arora *et al.* (2019) used RNA-Seq to identify genes associated with sheep muscle development (e.g., *SH3BGRL*), cellular stress (e.g., *HSPB1*), and fat metabolism (e.g., *ADIPOQ*, *FABP3*, *AACS*, and *TYSND1*). Genome-wide association studies have been used to screen genetic variants and candidate genes that influence economically important traits in various agricultural animals. In previous studies, SNPs associated with growth traits in sheep were identified by a genome-wide association study (GWAS). For example, Jiang J *et al.* (2021) conducted a GWAS analysis of the body size of 240 Hu sheep and found 5 SNPs associated with body height and 4 SNPs correlated with chest circumference. In 2020, Pasandideh *et al.* (2022) performed a GWAS of 96 Baluchi sheep using the 50K SNP-Chip to identify genetic variants associated with 8-mon body weight. *MTPN* is recognized as a candidate gene involved in the regulation of skeletal muscle growth. These studies provide a valuable list of genes and quantitative trait loci (QTLs) associated with improving the growth rate of sheep. Integrating GWAS and RNA-Seq is helpful for understanding the genetic architecture of economic traits in animals (Zhang *et al.* 2022; Wei *et al.* 2023). For instance, by combining GWAS and RNA-Seq data from 500 samples, Zhao *et al.* (2021) identified multiple candidate genes related to wool and growth traits in sheep. Li J *et al.* (2018) detected candidate genes and variants related to buffalo reproductive traits by integrating RNA-seq and GWAS. A genetic variant may modulate gene expression and thus affect complex phenotypes (Cloney 2016). Thus, integrating genomic variation and gene expression data can provide novel insights for better understanding the genetic mechanism architecture behind trait variation.

In this study, a combination of weighted gene coexpression network analysis (WGCNA) and RNA-seq was used to identify significant candidate genes and SNPs associated with the growth rate in sheep. WGCNA was then used to screen the hub genes that affect ADG. The tissue-specific genes (TSGs) were investigated in 10 tissues of Hu sheep, including the hypothalamus, rumen, jejunum, liver, lymph, lung, kidney, tail fat, muscle, and testis. In addition, the expression levels of the candidate genes in the hypothalamus of sheep with different growth rates were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot techniques. *BRINP3* and *PENK* polymorphisms and their association with the growth traits of 1,071 Hu sheep were further investigated. A major objective of this research was to identify valuable genes and genetic variants related to growth rates in sheep.

2. Materials and methods

2.1. Experimental animals and tissue sampling

All the sheep were raised in the same environment from weaning (56 d old) to 6 mon old at Minqin Defu Agriculture Co. Ltd. (Gansu, China), and they were fed during a 10 d pre-test period and a 100 d experimental period. The body weight of each sheep was measured and recorded in the morning on the 80, 100, 120, 140, 160, and 180 d of the experimental period. ADG was calculated as the total

Table 1 Growth traits of all the sheep used in RNA-Seq[1)]

Item	Hu sheep		Dorper sheep		P_1-value	P_2-value
	HF	HS	DF	DS		
No.	5	5	5	5		
ADG80–100 (kg)	0.36±0.02	0.27±0.01	0.27±0.05	0.10±0.03	0.006	0.017
ADG80–120 (kg)	0.35±0.02	0.28±0.02	0.31±0.03	0.14±0.03	0.043	0.007
ADG80–140 (kg)	0.36±0.02	0.30±0.03	0.31±0.03	0.18±0.03	0.107	0.014
ADG80–160 (kg)	0.34±0.02	0.29±0.03	0.32±0.02	0.20±0.03	0.167	0.013
ADG80–180 (kg)	0.34±0.02	0.26±0.03	0.30±0.02	0.20±0.02	0.039	0.012

[1)] ADG, average daily gain; HF, fast-growing Hu sheep; HS, slow-growing Hu sheep; DF, fast-growing Dorper sheep; DS, slow-growing Dorper sheep.

Each phenotypic value is shown as the mean±SE. P_1-value represents the HF *vs*. HS comparison; P_2-value is for the DF *vs*. DS comparison. *P*-values were calculated using a *t*-test.

body weight gained divided by days of the study. After measuring growth and development, 10 Hu sheep and 10 Dorper sheep were selected for RNA-seq according to their ADG at 80–180 d (Table 1). The experimental animals consisted of male lambs belonging to two sheep breeds but differing in growth rate: HF (Hu sheep, fast-growing, *n*=5; ADG80–180=0.34±0.02), HS (Hu sheep, slow-growing, *n*=5; ADG80–180=0.26±0.03), DF (Dorper sheep, fast-growing, *n*=5; ADG80–180=0.30±0.02), and DS (Dorper sheep, slow-growing, *n*=5; ADG80–180=0.20±0.02). Immediately after slaughter, samples of the *longissimus thoracis* tissue were taken from the hot carcass, frozen in liquid nitrogen, and stored frozen at –80°C until further analysis. The slaughter traits of Hu sheep used for RNA-Seq are shown in Appendix A.

2.2. Histological analyses

After slaughtering the sheep, *longissimus thoracis* samples were collected and immersed in 10% neutral formaldehyde overnight for fixation. Subsequently, the tissues underwent dehydration in ethanol solutions and then embedding in paraffin. Serial transverse sections were obtained by sectioning. The sections were examined using a digital trinocular camera microscope (BA210 Digital MacAudi Industrial Co., Ltd., China). Morphological characteristics of the muscle fibers, such as diameter, area, perimeter, and density, were measured. The software Motic Images Advanced 3.2 was used for image acquisition.

2.3. Genome wide association study

Based on the average daily gain (Fig. 2-A), HF (N_{case}=70, ADG80–180=0.32±0.004) and HS ($N_{control}$=70, ADG80–180=0.20±0.005) were used for the GWAS analysis. The slaughter traits of Hu sheep used for GWAS are presented in Appendix B. Blood samples (5 mL per sheep) were taken from the jugular vein of each individual at 180 d of age. Genomic DNA was extracted from whole blood samples with an *EasyPure* Blood Genomic DNA Kit (TransGen Biotech, Beijing, China). After conducting concentration and purity assays, high-quality DNA (with concentrations of >50 ng μL^{-1} and $1.8<A_{260}/A_{280}<2.0$) was used to construct a paired-end sequencing library. The library was then sequenced using the Illumina Novaseq PE150 platform (Illumina, San Diego, CA, USA).

High-quality sequencing data was guaranteed by eliminating paired reads of low quality. First, joint sequence reads were eliminated. Sequences were also filtered out if the number of undetected bases in single-end sequencing accounted for over 10% of the total sequence length. Lastly, bases with a quality value of Q≤5 and sequences comprising over 50% of the total length of the sequence were eliminated. After the filtering steps, all the samples were mapped against the Oar_rambouillet_v1.0 ovine genome using BWA (Burrows-Wheeler Aligner) with the parameters: "mem -t 4 -k 32 –M". SNP was called using the Bayesian method in the Genome Analysis Toolkit (GATK) from the BAM files. The following quality control steps were performed on the original variant dataset: (1) minor allele frequency (MAF) >5%; (2) missing rates <60%; (3) mean coverage depth ≥3; and (4) pruning for linkage disequilibrium (LD) (command: -indep-pairwise 100 10 0.5).

The fastGWA-GLMM (Jiang L *et al.* 2021) and mixed linear model (MLM) of GCTA software (Yang *et al.* 2011) were used to conduct the GWAS based on high-quality reads and accurate phenotypic records. The top three principal components (PCs) and batch effects of the genotype data were used as covariates. Principal component analysis (PCA) was performed on the pre-imputed genomic data using PLINK 1.90 (Purcell *et al.* 2007). The MLM was written as follows:

$$Y=W\alpha+G\beta+u+e$$

where Y represents the vector of phenotypes for fixed effects of the sheep, W is a matrix of fixed effects (including the top three PCs and batch effects), α is a vector of the corresponding coefficients; G is the matrix for genotypes based on all variants, while β is the relevant design matrices for G; u is the vector of random polygenic effects; and e stands for residual effects. Manhattan and Q-Q plots were created using the R software packages "qqman" and "CMplot", respectively. The genome-wide significance thresholds of MLM and fastGWA-GLMM were set as $-\log_{10}(1/\text{total SNPs})=6.52$ and $-\log_{10}(P\text{-value})=6$, respectively.

2.4. Library preparation and RNA-seq

Total RNA was isolated from the LT tissue of 10 Hu sheep (HF, HS, *n*=5 each) and 10 Dorper sheep (DF, DS, *n*=5 each) using TransZol (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 System (Agilent Technologies, CA, USA). RNA samples were stored at –80°C until RNA-Seq and qPCR analyses. The cDNA library was generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's protocol. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. The random hexamer and M-MuLV Reverse Transcriptase (RNase H-) were used as primers to synthesize the first-strand cDNA. The second-strand cDNA synthesis was subsequently performed with DNA Polymerase I and RNase H. To preferentially select cDNA fragments of 250–300 bp in length, the library fragments were purified using the AMPure XP system. The library preparations were sequenced using the Illumina Novaseq platform to generate paired-end reads of 150 bp in length.

2.5. RNA-seq quality control and differential expression analysis

Clean reads were obtained by removing the reads containing adapters, reads containing poly-N and low-quality reads from the raw data. All downstream analyses were based on the clean data of high quality. The clean reads were aligned to the ovine reference genome (https://www.ncbi.nlm.nih.gov/) using Hisat2 v2.0.5 software. The fragments per kilobase of transcript sequence per million (FPKM) base pairs sequenced values were used for quantifying gene expression levels.

Differential expression analysis of two groups was performed using the DESeq2 R package (1.16.1). Two criteria were used to determine DEGs: |fold change (FC)|>2 and *P*-value<0.05.

2.6. Weighted gene co-expression network analysis

The WGCNA (v1.72) package (Langfelder and Horvath 2008) in R (v4.3.1) was used to perform the weighted gene co-expression network analysis. Briefly, genes with expression>0.05 in at least 15 samples were selected for analysis. The "pickSoftThreshold" function was used to determine the optimal soft threshold power (β=1–20) for establishing a scale-free topology. The topological overlap matrix (TOM) was constructed using correlation expression values and used for the hierarchical clustering analysis of all genes. A total of eight traits of the Hu sheep and Dorper sheep were then correlated with gene expression levels to construct a systematic cluster tree. A heat map of the samples was created using the labeledHeatmap function. Using topology overlap measures, edge weights between different genes were calculated for the co-expression networks. Cytoscape software (v3.10.0) was used to visualize the networks.

2.7. Functional analysis of genes

GO enrichment analysis of genes was implemented by the clusterProfiler R package (Yu *et al.* 2012). KEGG is a database resource for understanding the high-level functions and utilities of biological systems (http://www.genome.jp/kegg/), and a *P*-value<0.05 was defined as indicating significantly enriched DEGs.

2.8. Integrative analysis of GWAS and RNA-seq

Genes associated with SNPs identified using GWAS were matched with the corresponding DEGs from the HF *vs*. HS and DF *vs*. DS comparisons. DEGs with the same expression pattern (co-up-regulated or co-down-regulated) between the HF *vs*. HS and DF *vs*. DS comparisons were identified. Venn diagrams are used to visualize these results.

2.9. Identification of tissue-specific genes

In this study, a total of 40 samples of RNA-Seq data from 10 tissues (hypothalamus, rumen, jejunum, liver, lymph, lung, kidney, tail fat, muscle, and testis) were used for TSG identification. These tissues were collected from four 6-mon-old male Hu sheep of similar weight. The RNA-Seq data were processed as described above. Three criteria were used for detecting tissue-specific genes (She *et al*. 2009; Tan *et al.* 2024): (1) The gene must be expressed at a non-zero level in at least one sample (FPKM≥1); (2) the average FPKM value of the candidate gene in a particular tissue must exceed that of other tissues by more than threefold; and (3) the average FPKM value of the candidate gene target tissue must be greater than 50% of the mean expression level in all other tissues.

2.10. Genomic DNA sequencing, SNP genotyping, and statistical analysis

To further investigate the *PENK* and *BRINP3* genes, specific primers were designed using Oligo 7.0 and then used to detect the SNP based on the available nucleotide sequence information (GenBank accession no. NC_040260.1 and NC_040263.1). Ten DNA pools were used for PCR amplification, and the PCR products were sequenced by Tsingke Biotechnology Co., Ltd. (Beijing, China). Based on accurate phenotypic records, genomic DNA from 1,071 male Hu sheep was used. The feeding and management conditions of these Hu sheep populations were consistent, as described above. Genotyping for two candidate SNPs within the *PENK* and *BRINP3* genes (*PENK* g.39289926 T>C, *BRINP3* g.16903465 T>C) was performed using competitive allele-specific FRET-based PCR analysis (KASPar) (Faucher *et al.* 2016). Information on the primers used in the KASPar analysis is presented in Appendix C. The Hardy-Weinberg equilibrium (HWE) was calculated using the SNPassoc v.2.1.0 (González *et al.* 2007) R package.

The correlations between traits and genotypes were analyzed using SPSS 20.0 statistical software. Animal models were defined as:

$$Y_{ijkl}=\mu+G_i+F_j+S_k+\varepsilon_{ijkl}$$

where the observed value of growth traits is represented by Y_{ijkl}. The mean is represented by μ, and the effects of the ith genotypes, jth farm (j=1, 2, 3), and kth season (k=1, 2) are represented by G_i, F_j, and S_k, respectively. The residual associated with the observed trait value is represented by ε_{ijkl}. A significance level of P<0.05 was used as the criterion for statistical significance.

2.11. Quantitative real-time PCR

The hypothalamus tissue of HF (n=5) and HS (n=5) Hu sheep were collected for qPCR. All growth traits of these sheep (ADG, body weight, body height, body length, chest circumference, and feed intake) are detailed in Appendix D. Total RNAs from the LT and hypothalamus tissues were extracted for qPCR as described above. Quantitative real-time PCR was performed using the SYBR premix *Ex Taq*™ Kit (TaKara Biotechnology, Japan) on a LightCycler 480 (Roche Applied Science, Mannheim, Germany). The reaction system had a volume of 20 μL: 2 μL of template cDNA, 0.8 μL of each primer, 10 μL of 2×SYBR Green PCR Master Mixture and 6.4 μL of RNase free water. The qPCR running conditions were as follows: 95°C for 3 min, 15 s at 95°C, and 20 s at 72°C, for 40 cycles. The sequences of the designed primers are listed in Appendix E. *ACTB* was used as an internal reference for normalizing target gene expression. Relative quantitative results were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.12. Protein extraction and Western blotting

A radioimmunoprecipitation-assay (RIPA) buffer (Servicebio, Wuhan, China) was used to homogenize and lyse the hypothalamus tissues for 30 min, followed by centrifugation at 12,000×g for 10 min at 4°C. The protein concentration in the supernatant was determined using the BCA Quantitative Test Kit (Servicebio, Wuhan, China). Following denaturation with 5× sodium dodecyl sulfate (SDS) buffer (Servicebio), protein samples were separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in Tris-buffered saline containing Tween 20 (TBST) for 2 h at 25°C, the membranes were incubated with primary antibodies against BMP/retinoic acid inducible neural specific

3 (BRINP3) (rabbit polyclonal antibody, ABclonal, 1:1,000), proenkephalin (PENK) (rabbit polyclonal antibody, ABclonal, 1:1,000), and β-actin (rabbit polyclonal antibody, Servicebio, 1:3,000) overnight at 4°C. After washing 3 times with TBST, the membranes were incubated with the HRP goat anti-rabbit secondary antibody (Servicebio, China; 1:3,000 dilution) for 1 h at 37°C. ImageJ software was used to measure the signals captured through film coloration.

3. Results

3.1. Comparison of morphological differences between the muscles of sheep with different ADG

The morphological observations of Hu sheep and Dorper sheep muscles at different ADG, using hematoxylin and eosin (H&E) staining, are presented in Fig. 1. As shown in Table 2, the diameter of the muscle fibers, the area of the muscle fibers, the perimysium thickness, and the perimeter of the muscle fibers in the HF group were significantly higher than those in the HS group (P<0.05). In addition, the density of muscle fibers in the HF group was significantly lower than that in the HS group (P<0.05). For Dorper sheep, the muscle fiber indicators of the DF group were greater than those of the DS group, but the differences were not significant (P>0.05).

3.2. Association analysis of ADG traits

After quality control, a total of 3,289,628 SNPs were retained for GWAS. Fig. 2-B and Appendix F show Q-Q plots for the GWAS analysis, which indicate no inflation or systematic bias. When MLM was applied, nine SNPs were found to be associated with the ADG of Hu sheep (Fig. 2-C). Based on the NCBI database, 10 genes were found within 50 kb distances from significant SNPs. The details of each gene are shown in Appendix G. Some of the identified genes are known to influence important economic traits related to the animals, including *BRINP3*, *PHYHIP*, and *LGI3*. Similarly, fastGWA-GLMM produced the same results by identifying the *LOC106990525*, *BRINP3*, and *DSCAML1* genes (Appendix F).

3.3. Analysis of RNA-Seq data and identification of DEGs

On average, totals of 445,404,056 and 465,846,280 raw reads were generated in the cDNA libraries of Hu sheep and Dorper sheep, respectively. The GC contents of the Hu sheep and Dorper sheep were 47.49–53.71% and 48.62–55.17%, respectively (Appendix H). The distributions of gene expression levels in different samples are shown in Appendix I. Based on the significance threshold criteria

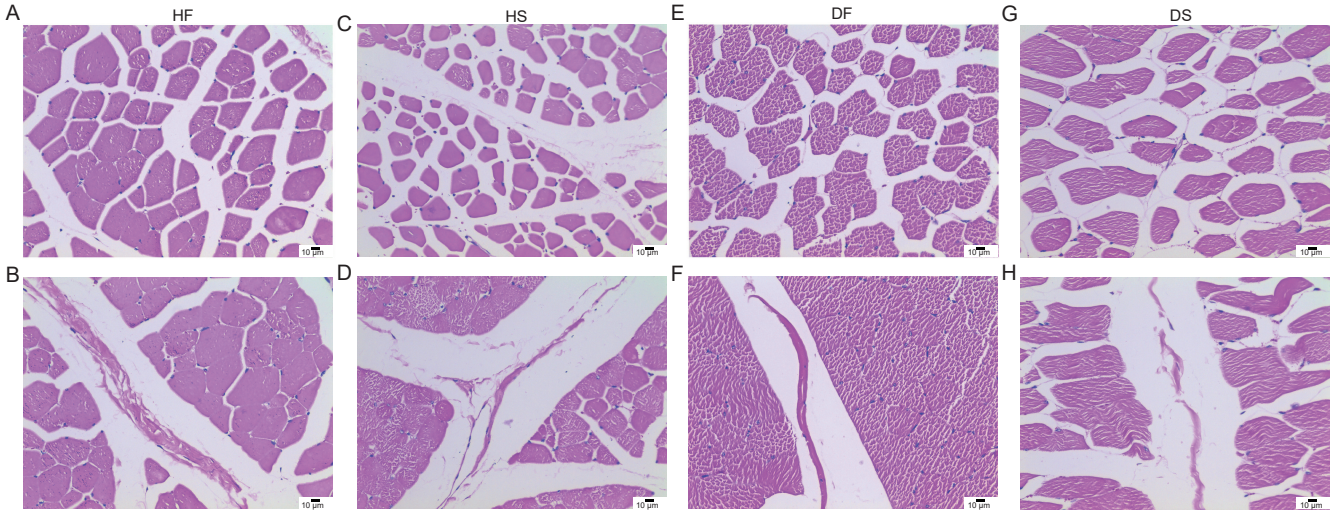


Fig. 1 Graphic of muscle fibers in sheep with different growth rates. A and B, hematoxylin and eosin (H&E) stained *longissimus thoracis* tissue sections of fast-growing Hu sheep (HF). C and D, H&E stained *longissimus thoracis* tissue sections of slow-growing Hu sheep (HS). E and F, H&E stained *longissimus thoracis* tissue sections of fast-growing Dorper sheep (DF). G and H, H&E stained *longissimus thoracis* tissue sections of slow-growing Dorper sheep (DS).

Table 2 Morphological comparisons of muscle tissues between Hu sheep and Dorper sheep with different average daily gain[1)]

Item	Hu sheep		Dorper sheep		P_1-value	P_2-value
	HF	HS	DF	DS		
No.	5	5	5	5		
Diameter of muscle fibers (μm)	35.69±0.82	32.12±1.27	37.18±0.73	35.37±0.79	0.021	0.097
Area of muscle fibers (μm^2)	1,616.36±65.28	1,363.08±89.16	1,958.39±83.70	1,795.48±55.42	0.024	0.108
Perimysium thickness (μm)	9.68±0.84	7.32±0.61	12.34±0.74	10.83±0.67	0.026	0.137
Perimeter of muscle fibers (μm)	152.75±3.08	139.59±4.39	172.96±4.24	166.62±3.15	0.016	0.233
Density of muscle fibers (fiber mm^{-2})	437.27±27.80	548.11±35.06	454.05±28.44	415.40±24.62	0.020	0.313

[1)] HF, fast-growing Hu sheep; HS, slow-growing Hu sheep; DF, fast-growing Dorper sheep; DS, slow-growing Dorper sheep.
Each phenotypic value is shown as the mean±SE. P_1-value represents the HF *vs*. HS comparison; P_2-value is the DF *vs*. DS comparison. P-values were calculated using a t-test.

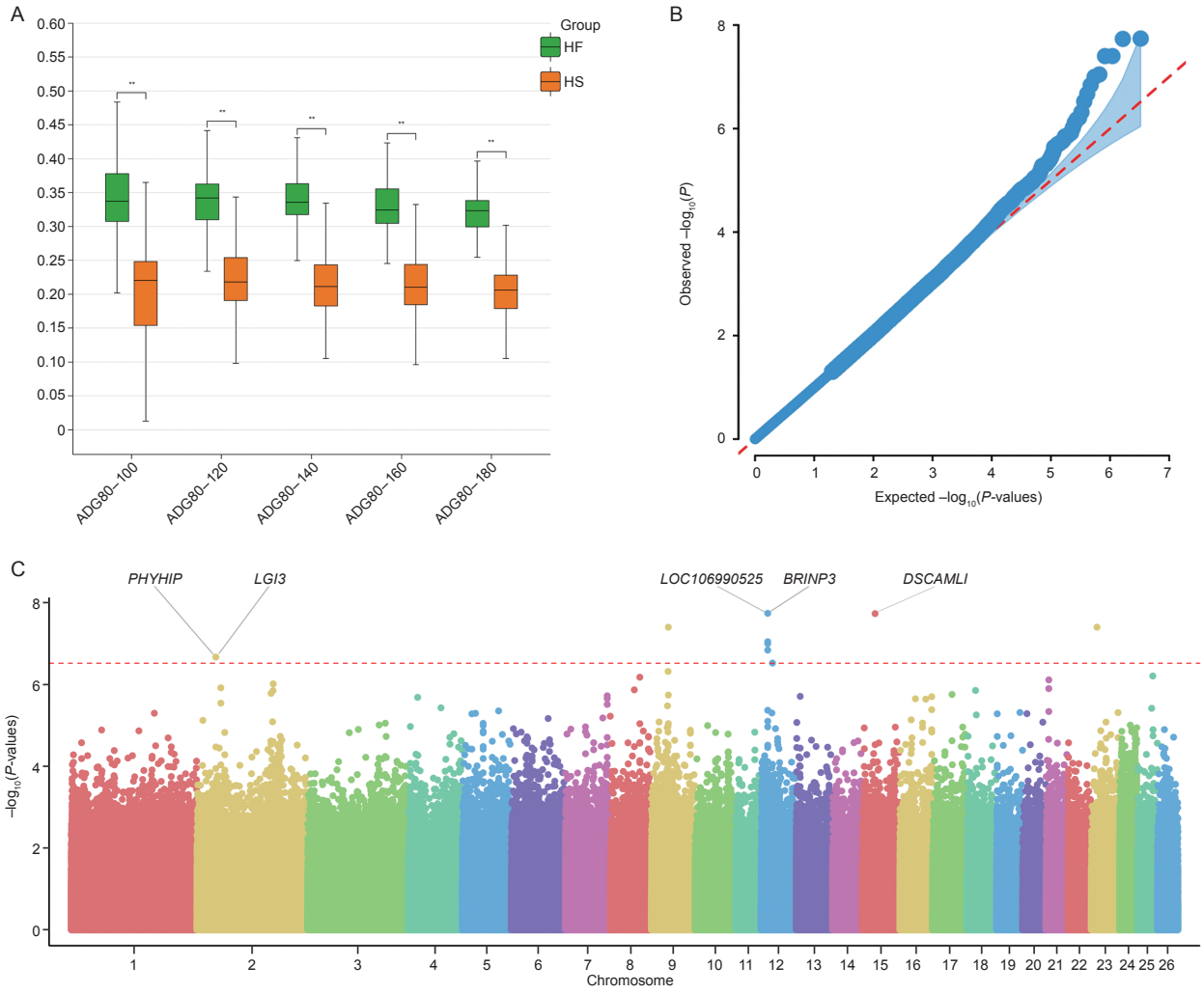


Fig. 2 Genome-wide association study (GWAS) of different growth rates in Hu sheep. A, average daily gain of the Hu sheep used for a GWAS. HF, fast-growing Hu sheep; HS, slow-growing Hu sheep; **, P<0.01. B, Q-Q plots of $-\log_{10}(P\text{-values})$ for average daily gain. C, Manhattan plots of average daily gain. The X-axis shows genomic positions with chromosome numbers, and the Y-axis shows $-\log_{10}(P\text{-values})$.

(|FC|>2 and P-value<0.05), a total of 501 DEGs were identified in the HF *vs*. HS comparison, with 211 up-regulated genes and 290 down-regulated genes (Fig. 3-A). Moreover, a total of 441 DEGs were identified in the DF *vs*. DS comparison, with 231 up-regulated genes and 210 down-regulated genes (Fig. 3-B). Hierarchical clustering shows the differences and similarities of DEGs in the HF *vs*. HS and DF *vs*. DS comparisons (Fig. 3-C and D). The results showed a significant difference in the gene expression profiles between the two comparisons.

qRT-PCR was used to assess the expression of DEGs to verify the reliability of the sequencing results. As shown in Appendix J, the results showed that the expression levels of the 10 genes in Hu sheep and Dorper sheep *longissimus thoracis* muscles were largely consistent with the RNA-Seq data (r=0.97). These results indicate that the sequencing data are reliable.

3.4. Functional enrichment analysis of DEGs

According to gene ontology, the DEGs detected in the HF *vs*. HS and DF *vs*. DS comparisons were significantly (P<0.05) enriched in 24 and 58 GO terms, respectively (Appendix K). In the HF *vs*. HS comparison, the DEGs were mainly distributed in the terms “electron transport chain”, “extracellular region”, “extracellular region part”, “actin cytoskeleton”, “calcium ion binding”, “molecular function regulator” and “DNA binding transcription factor activity” (Fig. 3-E). In the DF *vs*. DS comparison, most DEGs were distributed in “translation”, “peptide biosynthetic process”, “peptide metabolic process”, “ribonucleoprotein complex”, “ribosome”, “mitochondrion”, “structural constituent of ribosome”, “inorganic cation transmembrane transporter activity” and “cation transmembrane transporter activity” (Fig. 3-F).

A KEGG pathway analysis was conducted to investigate the genes and pathways in the *longissimus thoracis* of sheep related to growth traits. Totals of 35 and 14 pathways were identified in the DEGs of the HF *vs*. HS and DF *vs*. DS comparisons, respectively (Appendix L). The top 20 enriched pathways are shown in Fig. 3. In the HF *vs*. HS comparison, some of these pathways are related to fat metabolism, such

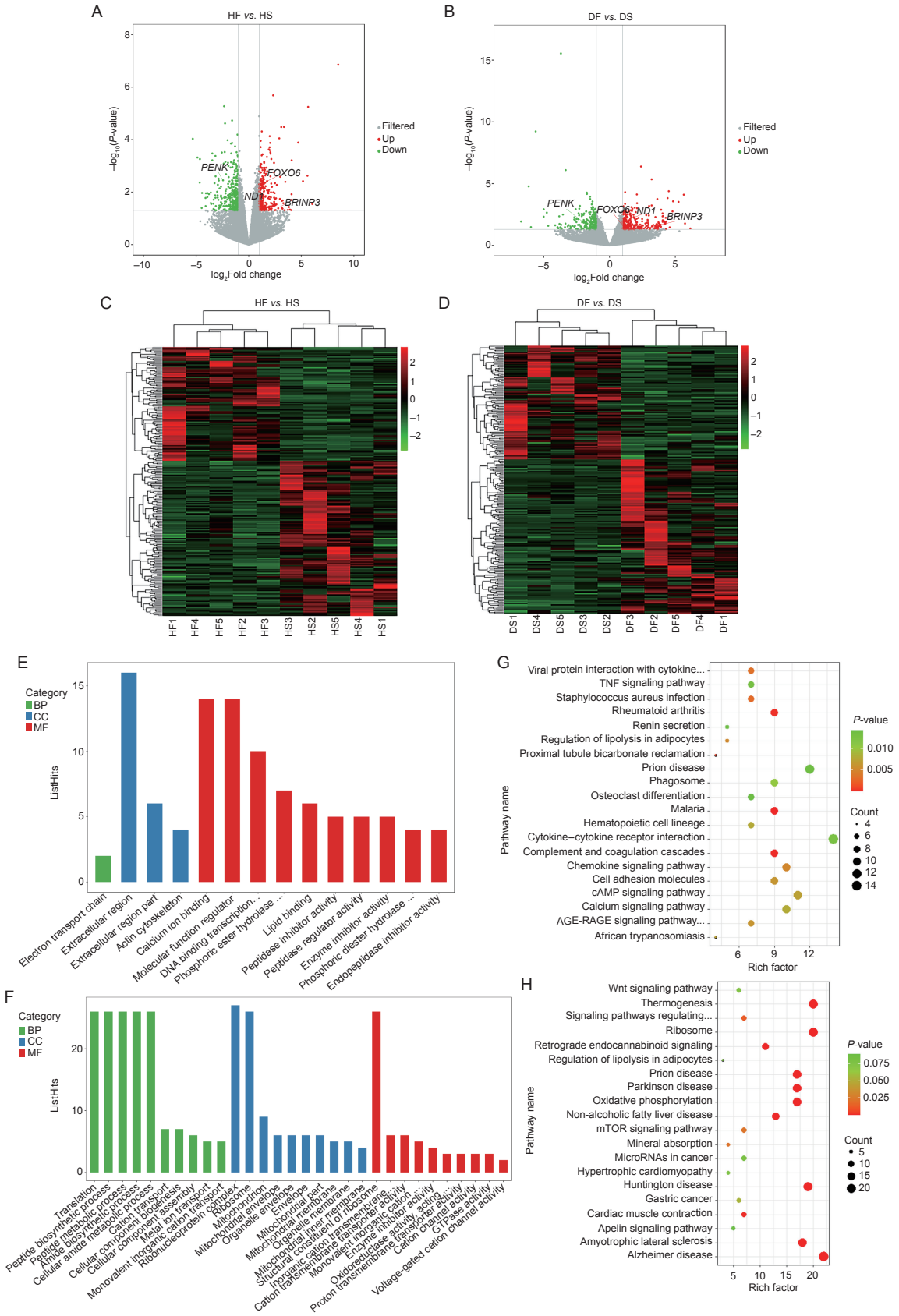


Fig. 3 Transcriptome sequencing analysis. A and B, volcano plot for up/down-regulated differentially expressed genes (DEGs) in the HF *vs*. HS and DF *vs*. DS comparisons, respectively. C and D, cluster analysis of DEGs in the fast-growing Hu sheep (HF) *vs*. slow-growing Hu sheep (HS) and fast-growing Dorper sheep (DF) *vs*. slow-growing Dorper sheep (DS) comparisons, respectively. E and F, gene ontology term enrichment for DEGs in the HF *vs*. HS and DF *vs*. DS comparisons, respectively. G and H, KEGG analysis of DEGs in the HF *vs*. HS and DF *vs*. DS comparisons, respectively.

as “regulation of lipolysis in adipocytes” and “cell adhesion molecules” (Fig. 3-G). In the DF *vs*. DS comparison, the significant enrichment pathways related to energy metabolism were “oxidative phosphorylation”, “thermogenesis” and “mineral absorption” (Fig. 3-H). In addition, two GO terms and two KEGG pathways were identified in both the HF *vs*. HS and DF *vs*. DS comparisons, including “electron transport chain”, “enzyme inhibitor activity”, “prion disease” and “mineral absorption” (Appendix M).

3.5. Identification of hub genes associated with ADG

Using the gene expression matrix obtained from the RNA-Seq data, a WGCNA analysis was conducted to identify the key gene clusters and hub genes associated with ADG in sheep (Fig. 4-A). As shown in Appendix I, 12 was found to be the optimal soft threshold. Appendix I shows the cluster tree diagram and network heatmap plot between the modules. Using *P*-value<0.05 as a threshold, the MEdarkolivegreen4 (r=0.47, P=0.04) and MEdarkmagenta (r=–0.54, P=0.02) modules were found to have a significant correlation with sheep ADG. In addition, the MEgreen module (r=0.45, P=0.06) displayed a trend toward significant correlations (Fig. 4-B and C). Based on the module membership (MM) and gene significance (GS) values of the genes, the top 5 genes in each module were identified as hub genes (such as *INPP5K*, *ZNF692*, *LRP3*, *PPP6R1*, *LOTAF*, and *PARVA*), and they were visualized with Cytoscape (Fig. 4-D).

3.6. Integrative analysis of the GWAS and RNA-Seq

An integrated analysis of DEGs identified in the HF *vs*. HS and DF *vs*. DS comparisons with GWAS signals revealed that the *BRINP3* gene was shared among all 3 groups (Fig. 5-A). As shown in Fig. 5-B, among the 28 DEGs that changed in the HF *vs*. HS and DF *vs*. DS comparisons, four DEGs showed the same expression patterns, including 1 co-down-regulated gene (*PENK*) and 3 co-up-regulated genes (*ND1*, *BRINP3*, and *FOXO6*). These 4 DEGs are described in detail in Appendix N. Furthermore, a negative association was found between *PENK* gene expression levels and ADG as well as average daily feed intake (ADFI) in sheep at each stage. Conversely, the *ND1*, *BRINP3*, and *FOXO6* genes exhibited a positive associations (Fig. 5-C).

3.7. Tissue-specific gene detection

A total of 23,549 genes expressed in at least one sample were retained to identify the tissue-specific genes. The principal component analysis (PCA) of the 10 tissues is shown in Fig. 6-A. This analysis identified the TSGs that overlap with the DEGs in the HF *vs*. HS and DF *vs*. DS comparisons (Fig. 6-B, Appendix O). Notably, the *BRINP3* gene is one of the TSGs of the hypothalamus and shows the highest expression in the hypothalamus (FPKM=5.02, Fig. 6-B and D). However, the *PENK* gene was not present in the identified TSGs, and it showed the highest expression in tail fat (FPKM=44.24), followed by the hypothalamus (FPKM=11.94, Fig. 6-C). The expression patterns of DEGs that overlap with TSGs belonging to muscle and their correlation with ADG were investigated. In the HF *vs*. HS comparison, 13 up-regulated DEGs showed a positive correlation with ADG, and the correlations increased with age (Fig. 6-E). In the DF *vs*. DS comparison, 12 up-regulated DEGs correlated positively with ADG and 5 down-regulated DEGs correlated negatively (Fig. 6-F). These results provide further evidence for the importance of identifying TSGs.

3.8. Genotyping and association analysis of candidate genes

A novel missense mutation (*PENK* g.39289926 T>C) located in exon 1 of the *PENK* gene was identified in the Hu sheep population (Fig. 7-A). A total of 1,071 individuals were successfully genotyped, and 3 genotypes were identified: CC, CT, and TT (Fig. 7-B). A novel synonymous mutation (*BRINP3* g.16903465 T>C) within exon 7 of *BRINP3* was also identified in the Hu sheep population (Fig. 7-C). The genotyping of 999 individuals revealed 3 genotypes: CC, TC, and TT (Fig. 7-D). For the *PENK* g.39289926 T>C loci, the CT genotype is present at a higher frequency (0.49) than the CC (0.24) and TT (0.27) genotypes. For the *BRINP3* g.16903465 T>C loci, the genotypic frequencies of CC, TC, and TT were 0.63, 0.34, and 0.03, respectively. A Hardy-Weinberg equilibrium test indicated that these two SNPs were in HWE (P>0.05, Appendix P).

The results of the association analysis indicated that the *PENK* g.39289926 T>C polymorphism was significantly associated with the body weight of sheep. Individuals with the CC genotype had a higher body weight than those with the TT genotype (P<0.05, Table 3). Meanwhile, the *BRINP3* g.16903465 T>C polymorphism exhibited a significant association with sheep body weight. Sheep carrying the CC genotype had significantly higher body weights at 80, 100, 120, 140, 160, and 180 d compared to those with the TT genotype (P<0.05, Table 4). For the feed intake trait, the differences between genotypes at the 2 loci and phenotypes were not significant (P>0.05), but they exhibited the same trend as body weight.

3.9. qRT-PCR and Western blot analysis

To further investigate the functions of *BRINP3* and *PENK*, their expression levels were assessed in the hypothalamus tissues of sheep with different growth rates using qRT-PCR and Western blot techniques. As shown in Fig. 7-E, the qRT-PCR results showed that the expression level of *BRINP3* in the HF group was significantly higher than in the HS group (P<0.01), while the expression level of *PENK* was significantly higher in the HS group than in the HF group (P<0.05). The BRINP3 protein level in the HF group was significantly higher than in the HS group (P<0.05, Fig. 7-F and G). However, there was no significant difference in the expression levels of PENK protein between the HF and HS groups (P>0.05, Fig. 7-F and H).

4. Discussion

Growth traits are major factors that affect the economic productivity of livestock, and they are controlled by several major genes and multiple minor genes (Ruan *et al.* 2021). ADG is an important indicator for measuring the growth rate

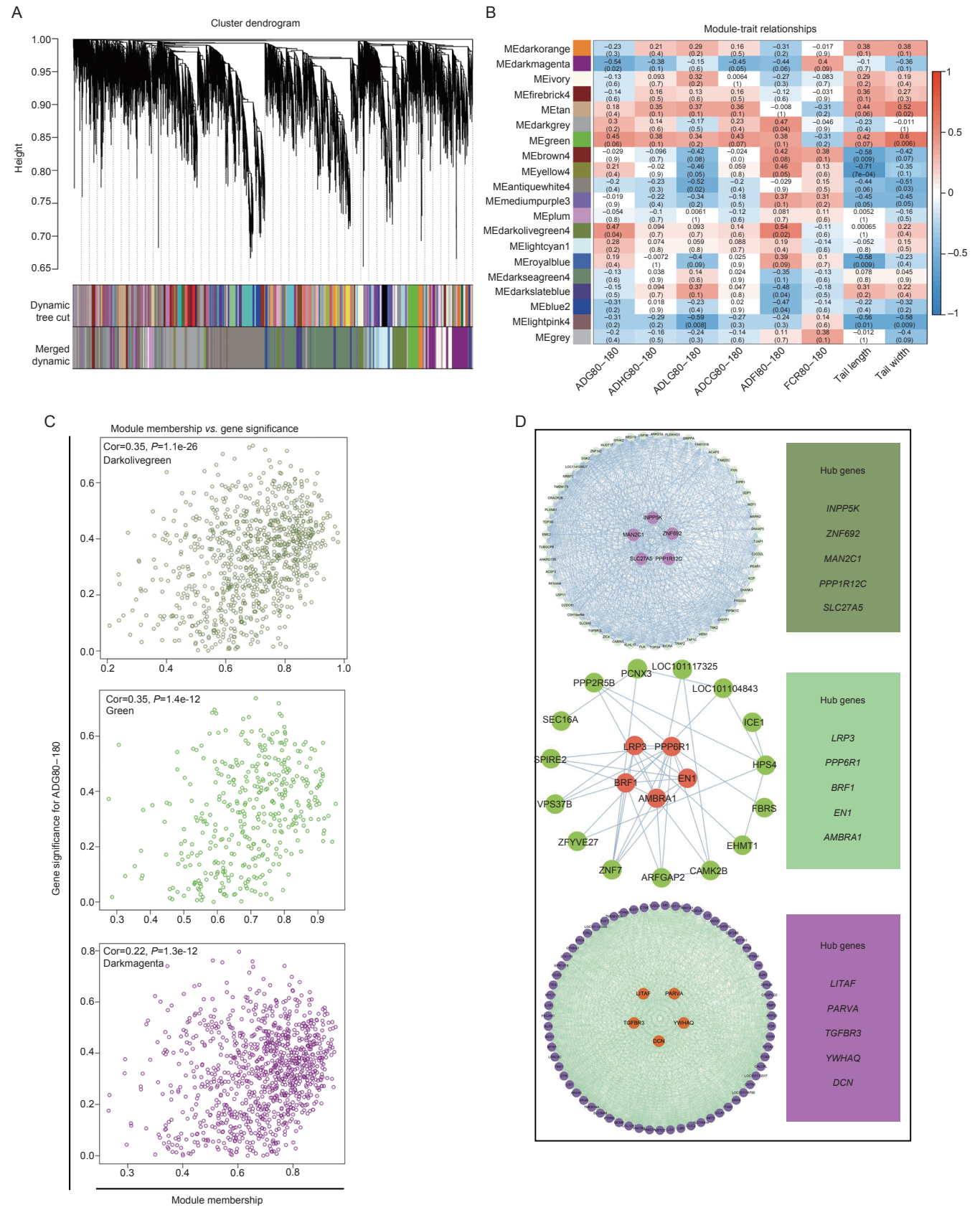


Fig. 4 Weighted gene co-expression network analysis of genes. A, cluster dendrogram of the co-expression network modules (1-TOM). B, correlations between the module eigengenes and phenotypes, and the *P*-values are shown. ADG, average daily gain; ADHG, average daily height gain; ADLG, average daily lean gain; ADCG, average daily carcass gain; ADFI, average daily feed intake; FCR, feed conversion ratio. C, scatter plot analysis of 3 related modules. D, hub gene interaction network of in the 3 modules. The hub genes are shown separately.

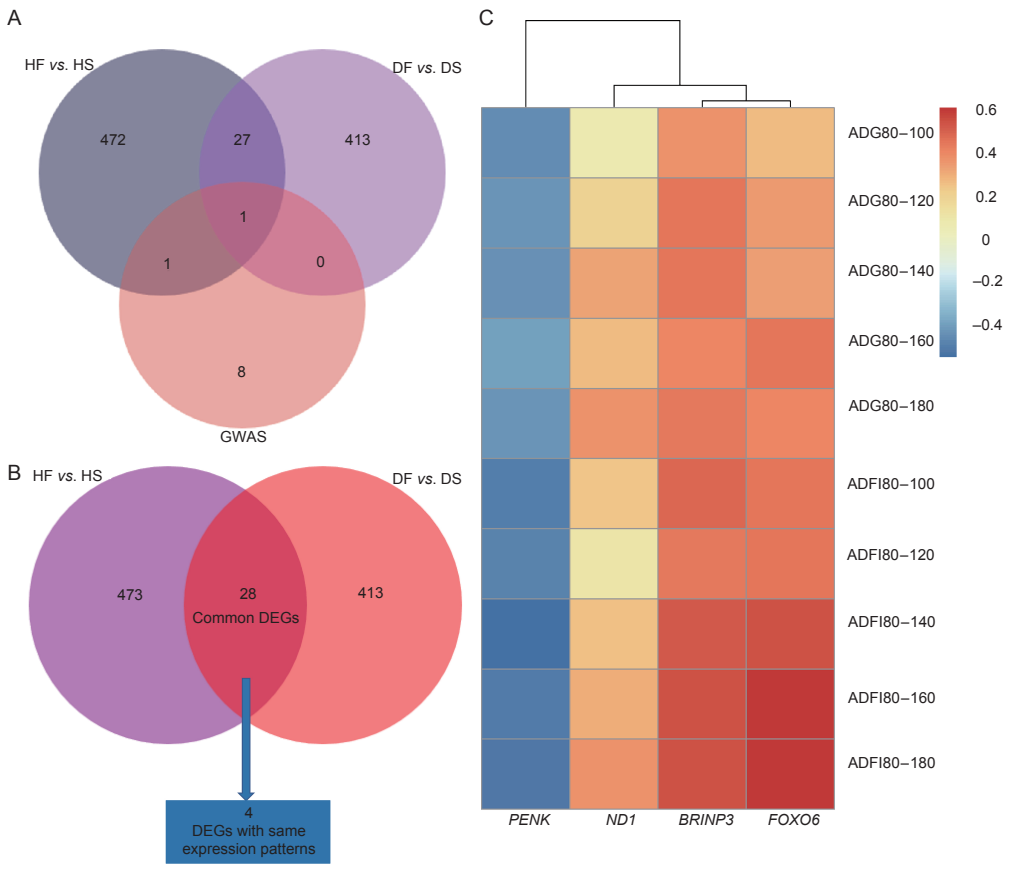


Fig. 5 Integrative analysis of transcriptome and genome-wide association study (GWAS) data. A, Venn diagram of the RNA-Seq and GWAS data. B, Venn diagram of differentially expressed genes (DEGs) between the fast-growing Hu sheep (HF) *vs*. slow-growing Hu sheep (HS) and fast-growing Dorper sheep (DF) *vs*. slow-growing Dorper sheep (DS) comparisons, with the 4 DEGs showing the same expression pattern. C, correlation analysis of the 4 DEGs exhibiting the same expression pattern with average daily gain (ADG) and average daily feed intake (ADFI).

of animals (Fontanesi *et al*. 2014). In this study, the body weights of Hu sheep and Dorper sheep were measured and recorded at 6 stages (80, 100, 120, 140, 160, and 180 d of age), and the ADG was calculated. Differences in ADG exist between the HF *vs*. DF groups and the HS *vs*. DS groups. In addition, differences in muscle fiber indicators were found among sheep with different growth rates, although the differences were not significant in Dorper sheep. The rate of growth can lead to alterations in muscle fiber characteristics (Remignon *et al*. 1995). It is commonly accepted that the number of muscle fibers is determined by genetics before birth, and after that, only the length and cross-sectional area of the muscle fibers increase (Stickland *et al*. 1975). Genetically, growth rate was positively correlated with fiber cross-sectional areas (Larzul *et al*. 1997). In this study, fast-growing sheep exhibited larger muscle fiber diameter, area, perimeter, and perimysium thickness compared to slow-growing sheep. A genome-wide association study of Hu sheep populations with different ADG identified several genes associated with growth rate, including *BRINP3*, *PHYHIP*, and *LGI3*. Studies have found that *PHYHIP* is one of the candidate genes associated with 6-mon body weight in Zandi sheep (Mohammadi and Rafat 2020), and there is evidence indicating that LGI3 may serve depot-specific roles as an adipokine in the adipose tissues of mice (Kim *et al*. 2021). Fat deposition and muscle growth are known to determine the body weight of animals. A higher growth rate may result in increased fat deposition (Na *et al*. 2020). Therefore, it is not surprising that these identified genes are associated with fat deposition.

Totals of 501 and 441 DEGs were identified by comparing the transcriptomes of *longissimus thoracis* tissue in the HF *vs*. HS and DF *vs*. DS comparisons, respectively. In the HF *vs*. HS comparison, the functional analysis results showed that some pathways are associated with fat synthesis and metabolism, such as “regulation of lipolysis in adipocytes” and “cell adhesion molecules”. Some of the genes enriched in these pathways have been previously identified as key genes affecting animal fat deposition, including the *ADRB3*, *PDE3B*, *VCAN*, *FABP4*, and *SERPINE1* genes. In Hu and Altay sheep, the adrenoceptor beta 3 (*ADRB3*) gene has been identified as a candidate gene for thermogenesis (Jiao *et al*. 2021), energy regulation, and lipolysis (Wu *et al*. 2012), and allelic variation at the *ADRB3* locus was associated with carcass fat in sheep (Forrest *et al*. 2003). Loss of function of the *PDE3B* gene is associated with an increased gluteofemoral fat mass in humans (Mine *et al*. 2021). As in previous studies, the *PDE3B* gene was up-regulated in the slow-growing group in this study, suggesting a potential



Fig. 6 Study of tissue-specific genes. A, principal component analysis for 10 tissue types. The experiment included 4 biological replicates for each tissue. B, tissue-specific gene expression patterns in 10 tissues. Each tissue has unique coloring. All differentially expressed genes (DEGs) that overlap with tissue-specific genes (TSGs) are shown on the right. C, expression levels of the *PENK* gene in 10 tissues. D, expression levels of the *BRINP3* gene in 10 tissues. E, expression patterns of DEGs (in the fast-growing Hu sheep (HF) *vs*. slow-growing Hu sheep (HS) comparison) overlapping with TSGs belonging to muscle and their correlations with average daily gain (ADG). F, expression patterns of DEGs (in the fast-growing Dorper sheep (DF) *vs*. slow-growing Dorper sheep (DS) comparison) overlapping with TSGs belonging to muscle and their correlations with ADG.

negative correlation with fat accumulation in sheep. In addition, the *PDE3B* gene may be an important regulator of energy metabolism in adipose tissue and is also related to the treatment of obesity (Chung *et al.* 2017). The *VCAN* gene was identified as a candidate gene for pork quality traits and fat deposition by a comprehensive transcriptome analysis (Piórkowska *et al.* 2018). In addition, Wang *et al.* (2021) demonstrated that the *VCAN* gene is associated with fat metabolism in chickens. Fatty acid binding protein 4 (FABP4) is a member of the FA-binding protein family. Several studies

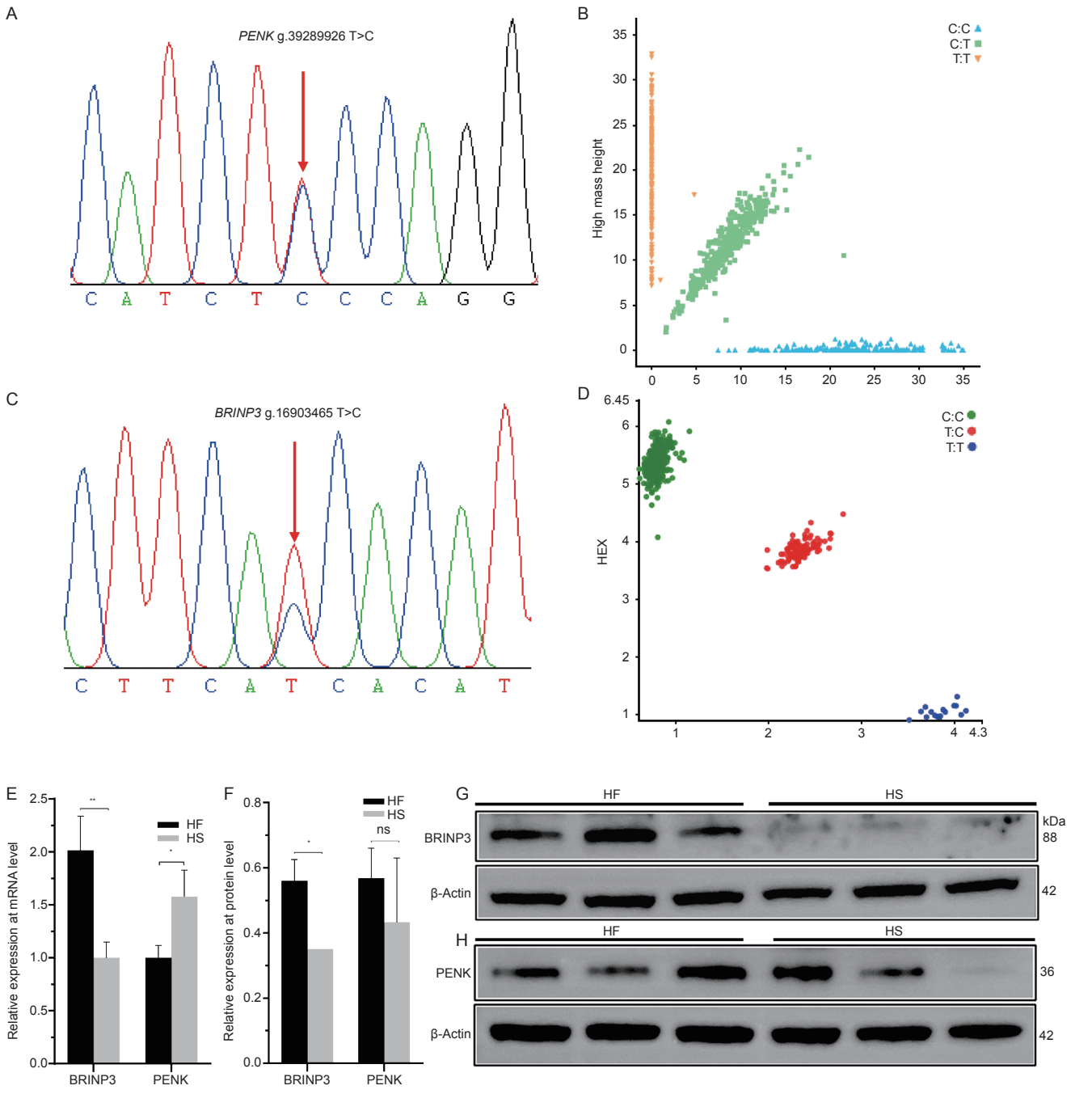


Fig. 7 Gene functional analysis of *BRINP3* and *PENK*. A, image of the sequencing peaks of *PENK* g.39289926 T>C. B, Kaspar-based SNP genotyping of *PENK* g.39289926 T>C. C, image of the sequencing peaks of *BRINP3* g.16903465 T>C. D, Kaspar-based SNP genotyping of *BRINP3* g.16903465 T>C. E, mRNA expression levels of *BRINP3* and *PENK*. HF, fast-growing Hu sheep; HS, slow-growing Hu sheep; *, $P<0.05$; **, $P<0.01$. F, protein expression levels of BRINP3 and PENK. *, $P<0.05$; **, $P<0.01$. G, expression patterns of the control gene β-actin and target gene BRINP3 in the hypothalamus examined by Western blot analysis. H, expression pattern of the control gene β-actin and target gene PENK in the hypothalamus examined by Western blot analysis.

have shown that variations in the *FABP4* gene are associated with carcass growth (Yan *et al.* 2018; Shafey *et al.* 2020), meat quality (Tao *et al.* 2011), and fat deposition traits in sheep. Similar reports have been documented in other animals (Ballester *et al.* 2017; Goszczynski *et al.* 2017; Yin *et al.* 2020). Thus, these genes may influence the growth rate of Hu sheep by regulating their fat deposition.

In the DF *vs*. DS comparison, the KEGG pathways of the DEGs related to energy metabolism were significantly enriched, such as Thermogenesis (*PLIN1* and *PPARGC1A* genes) and Cardiac muscle contraction (*MYL3* gene). *PLIN1* (Perilipin 1) was shown to be a candidate gene for the regulation of intramuscular fat (IMF) content in pigs (Li B *et al.* 2018). Furthermore, overexpression of *PLIN1* promotes lipid metabolism in bovine adipocytes (Li *et al.* 2020) and *PLIN1* deficiency results in reduced fat mass in mice (Sohn *et al.* 2018). In this study, the *PLIN1* and *PPARGC1A* genes were significantly up-regulated in the DF group. Skeletal muscles

Table 3 Association analysis between the growth traits and polymorphism of the *PENK* and *BRINP3* genes

Locus	Trait[1)]	Genotypes (mean±SE)		
		CC (262)	TC (523)	TT (286)
PENK g.39289926 T>C	BW80	19.943±0.254 a	19.490±0.175 a	18.810±0.229 b
	BW100	25.161±0.312 a	24.564±0.207 a	23.944±0.279 b
	BW120	30.923±0.361 a	30.350±0.225 ab	29.903±0.305 b
	BW140	36.701±0.384 a	36.036±0.246 ab	35.545±0.318 b
	BW160	42.601±0.414 a	41.829±0.260 ab	41.272±0.342 b
	BW180	47.432±0.425 a	46.819±0.274 ab	46.174±0.362 b
	FI80–180	166.123±1.684	165.330±1.121	163.970±1.533
	FI100–180	142.436±1.411	142.239±0.928	141.307±1.276
	FI120–180	112.504±1.086	112.060±0.728	111.324±0.967
	FI140–180	77.807±0.741	77.579±0.515	76.981±0.668
	FI160–180	39.569±0.398	39.866±0.272	39.544±0.360
Locus	Trait[1)]	Genotypes (mean±SE)		
		CC (630)	TC (335)	TT (34)
BRINP3 g.16903465 T>C	BW80	18.299±0.170 a	18.303±0.234 a	16.908±0.733 b
	BW100	22.747±0.218 a	22.680±0.299 a	21.135±0.939 b
	BW120	28.041±0.259 a	27.865±0.355 a	25.844±1.114 b
	BW140	33.674±0.289 a	33.408±0.396 a	31.188±1.244 b
	BW160	39.100±0.316 a	38.823±0.433 a	36.491±1.359 b
	BW180	43.844±0.325 a	43.378±0.446 ab	41.176±1.401 b
	FI80–180	164.187±1.340	163.034±1.881	157.770±7.377
	FI100–180	142.080±1.117	140.749±1.588	136.744±6.160
	FI120–180	112.954±0.869	111.968±1.229	109.276±4.676
	FI140–180	78.094±0.604	77.508±0.854	75.922±3.088
	FI160–180	39.805±0.319	39.372±0.443	38.683±1.515

[1)] BW, body weight; FI, feed intake.
Each phenotypic value is shown as the mean±SE. Values with the same lower-case letters in the same line show no significant difference (P>0.05), while those with different letters show a significant difference (P<0.05).

play indispensable roles in body motion and the maintenance of metabolism (Rakus *et al.* 2015). PPARG coactivator 1 alpha (*PPARGC1A*) is a key transcriptional coactivator of *PPARG* in response to environmental stimuli, and it plays key roles in several metabolic pathways related to energy homeostasis, gluconeogenesis, and adipogenesis in muscle and adipose tissue (Pere and Spiegelman 2003). Studies have shown that the *PPARGC1A* gene is related to the hot carcass weight and carcass fat content of sheep (Armstrong *et al.* 2018). In addition, the *MYL3* gene responds to differentiation and growth performance in quail muscle (Park *et al.* 2018) and is considered a candidate gene for chicken embryonic muscle development (Ouyang *et al.* 2017). MYL3 is considered a key protein involved in muscle growth, contraction, and lipid deposition in pigs (Ropka-Molik *et al.* 2015; Wang *et al.* 2017). These genes have the potential to serve as candidate genes associated with the growth rate of Dorper sheep. The use of WGCNA is advantageous not only for establishing a gene regulatory network but also for identifying hub genes within that network (Zhang *et al.* 2022). This method has been successfully employed to identify the candidate genes related to important traits of sheep (He *et al.* 2022). This study identified three co-expression modules associated with ADG by WGCNA, and several hub genes, such as *INPP5K*, *SLC27A5*, *LITAF*, and *TGFBR3*, within these modules. Mutations in the *INPP5K* gene cause a congenital muscular dystrophy syndrome, which is highly expressed in muscle (Osborn *et al.* 2017). A study by Sui *et al.* (2023) found that *SLC27A5* is enriched in the PPAR signaling pathway and may contribute to IMF deposition in Ningxiang pigs. In obese pigs, *LITAF* mRNA isoform expression was higher than in pigs with less fat deposition (Cardoso *et al.* 2018). *TGFBR3* was found to be related to muscle cell development and differentiation (Cheng *et al.* 2020). Thus, these hub genes may play essential roles in muscle development and fat deposition in sheep.

Notably, 4 DEGs showed the same expression patterns in the two comparisons in this study, including 3 up-regulated genes (*FOXO6*, *BRINP3*, and *ND1*), and 1 down-regulated gene (*PENK*). These DEGs with the same expression pattern may play an important role, so they deserve more attention. *FOXO6* (forkhead box O6) is a member of the *forkhead box O* (*FoxO*) subfamily, and it is reported to affect chicken obesity by controlling preadipocyte proliferation, apoptosis, and early adipogenesis (Abdalla *et al.* 2021). Moreover, haplotypes of bovine *FOXO6* gene sequence variants are associated with growth traits in cattle (Sun *et al.* 2013). Another up-regulated gene is *BRINP3*, which was detected in both GWAS and the two comparisons, and it may play a crucial role in sheep growth. Qiu *et al.* (2018) identified important candidate genes in growth-related QTL regions, including *BRINP3*. Similarly, Xia *et al.* (2016) identified the *BRINP3* gene as a candidate gene for meat quality traits in beef cattle by GWAS. In addition, the SNP in *BRINP3* is associated with early embryonic development in Korean cattle (Lee *et al.* 2020).

Within the DEGs with the same expression patterns, the *PENK* (proenkephalin) gene was downregulated in both comparisons in this study. Previous studies have shown that the *PENK* gene affects the weaning weight of pigs (Jiao *et al.* 2014) and the height of cattle (Utsunomiya *et al.* 2013; An *et al.* 2019). The hypothalamus plays a crucial role in integrating the regulation of feeding behavior in mammals (Innes *et al.* 2023). Interestingly, we demonstrated that *BRINP3* is a tissue-specific gene in the hypothalamus, and *PENK* was found to be expressed at higher levels in the hypothalamus and adipose tissue compared to other tissues. Würfel *et al.* (2022) suggests that the *PENK* gene affects the development of obesity by directly modifying individual eating behavior and is negatively correlated with the human body mass index (BMI). In the hypothalamus of food-deprived mice, the mRNA level of *PENK* is significantly reduced (Ikeda *et al.* 2015). Furthermore, mice lacking the *Brinp3* gene exhibit marked anxiety responses and reduced body mass (Berkowicz *et al.* 2016). The results of this study revealed that the expression levels of *BRINP3* correlate positively with ADG and ADFI, whereas *PENK* correlates negatively. Moreover, the results of the association analysis indicated a significant association between polymorphisms in these two genes and body weight in sheep. Therefore, we speculate that *BRINP3* and *PENK* further influence growth rates by regulating feeding behavior in sheep. The qPCR and Western blot results of this study support this hypothesis. Nonetheless, future work will be needed to verify the associations between these identified candidate genes and growth rates in sheep.

5. Conclusion

In summary, totals of 501 and 441 DEGs were identified in the HF *vs*. HS and DF *vs*. DS comparisons, respectively. Some DEGs related to fat deposition and muscle development were observed in the *longissimus thoracis* of sheep, including *ADRB3*, *PDE3B*, *FABP4*, *VCAN*, *SERPINE1*, *PLIN1*, *PPARGC1A*, *MYL3*, and *FOXO6*. Through weighted gene co-expression network analysis, several hub genes (such as *INPP5K*, *SLC27A5*, *LITAF*, and *TGFBR3*) were screened. Furthermore, the integration of GWAS and RNA-Seq data highlighted *BRINP3* and *PENK* as key candidate genes affecting the growth rate in sheep. These findings provide a better understanding of the mechanisms affecting the growth rate in sheep, and the identified candidate genes provide valuable insights for future functional characterization.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

In this study, all animal studies and experimental procedures were consistent with the guidelines of the government of Gansu People's Congress, China. The experimental protocols were reviewed and approved by the Animal Care Committee of Lanzhou University, China (2011-01 and 2011-02).

Data availability

The RNA-Seq data of 10 Hu sheep and 10 Dorper sheep obtained in the study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA785419, and the RNA-Seq data for the 10 tissues reported in this study are available upon request for research purposes. The whole-genome sequences of 140 Hu sheep have been deposited to the NCBI (PRJNA1122090).

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2024.08.011

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