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Characterization of pig skeletal muscle transcriptomes in response to low temperature

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Abstract

Objectives: The response of mammals to cold environment is a complex physiological activity, and its underlying mechanism must be analyzed from multiple perspectives. Skeletal muscle is an important thermogenic tissue that maintains body temperature in mammals. We dissected the molecular mechanism of pig skeletal muscle response to a cold environment by performing comparative transcriptome analysis in the Enshi black pig.

Methods: Three pigs were subjected to acute cold stress (3 days), three pigs were subjected to cold acclimation (58 days), and three pigs were used as controls. RNA-seq was used to screen the differentially expressed genes (DEGs) of skeletal muscle.

Results: Using RNA-seq methods, we identified 1241 DEGs within the acute cold stress group and 1886 DEGs within the cold acclimation group. Prolonged cold exposure induced more gene expression changes. A total of 540 key cold-responsive DEGs were found, and their trends were consistent within the acute cold stress group and cold acclimation group. Gene expression pattern analysis showed that there were significant differences between the low-temperature treatment groups and the control group, and there were also differences between individuals after long-term low-temperature treatment. Analysis of DEGs revealed that 134 pathways were significantly enriched in the cold adaptation group, 98 pathways were significantly enriched in the acute cold stress group, and 71 pathways were shared between the two groups. The 71 shared pathways were mainly related to lipid, amino acid, and carbohydrate metabolism; signal transduction; endocrine, immune, and nervous system; cardiovascular disease; infectious diseases caused by bacteria or viruses; and neurodegenerative disease.

Conclusions: In conclusion, this study provides insights into the molecular mechanism of porcine skeletal muscle response under low-temperature environment. The data may assist further research on the mechanism of pig response to cold exposure.

KEYWORDS
Enshi black pig, low temperature, RNA-seq, DEGs, pathway

1 | INTRODUCTION

The pig (*Sus scrofa*) is a commonly bred livestock animal that provides energy, protein, and micronutrients for human consumption. The intensive production of pigs has resulted in their sensitivity to environmental stress. Although livestock and poultry are protected by humans, they are not as highly exposed to environmental factors as crop plants. Recurring extreme weather caused by global climate change can negatively affect pig production because pigs are more sensitive to temperature extremes than cattle and sheep (Berg et al., 2006).

Pigs are especially sensitive to low-temperature environments. Pigs lack the uncoupling protein gene *UCP1* which prevents them from producing brown adipose tissue (BAT) (Hou et al., 2017). As a result, pigs cannot maintain body temperature through nonshivering thermogenesis of brown fat, as can other mammals. Pigs may maintain body temperature through muscle shivering and nonshivering thermogenesis as in UCP1-deficient birds (Nowack et al., 2017). Cold stress is recognized as a main cause of pig neonatal morbidity and mortality. A possible explanation is the inability of pigs to generate a febrile response (Carroll et al., 2012). Acute physiological responses to cold exposure include cutaneous vasoconstriction and shivering thermogenesis, which help to decrease heat loss and increase metabolic heat production (Castellani & Young, 2016).

Skeletal muscle and BAT are the main thermogenic tissues of the body. At room temperature, the heat production of skeletal muscle accounts for about 20%, but in a cold environment, its heat production can increase to 40% (Blondin & Haman, 2018). Skeletal muscle provides heat to the body in both shivering and nonshivering thermogenesis, which is especially important for large mammals with less brown fat or birds and pigs without BAT. The shivering thermogenesis of skeletal muscle is mainly through the involuntary rhythmic contraction of skeletal muscle, which increases the metabolic rate and heat production. Nonshivering thermogenesis of skeletal muscle is independent of shivering thermogenesis, and mitochondrial proton leak and sarcoplasmic reticulum calcium cycle are known to be the main thermogenesis mechanisms.

Research reports on muscle heat production first appeared in 1954. Sellers et al. (1954) proposed that in a cold environment, the skeletal muscle of mice maintains body temperature through electrical activity used to generate heat. Low-temperature environments can lead to increased oxygen consumption of skeletal muscle. Removal of intramuscular nerves does not affect muscle heat production and nonshivering heat production of muscle may be humoral mediated (Davis, 1967). The Ca^{2+}-ATPase (Sarcoplasmic reticulum Ca^{2+}-ATPase, *SERCA*) on the sarcoplasmic reticulum membrane can mediate the pumping of Ca^{2+} from the cell matrix into the sarcoplasmic reticulum and store it in the sarcoplasmic reticulum. The Ca^{2+} concentration in the plasmic reticulum is much higher than that in the cytoplasm. When the body is stimulated by nerve impulses or environmental changes, Ca^{2+} is released from the high-concentration sarcoplasmic reticulum cavity into the cytoplasm. During this process, part of Ca^{2+} is coupled with the synthesis of ATP, and part of Ca^{2+} leaks and deviates from ATP. It is synthesized and leads to heat loss (De Meis et al., 1997). During mild and severe cold adaptation, nonshivering thermogenesis of muscle and BAT are simultaneously activated. If one pathway is blocked, the other pathway increases thermogenesis to maintain the core temperature and the two pathways interact with each other (Bal et al., 2017). In addition, mitochondrial crosstalk in the sarcoplasmic reticulum, increased mitochondrial biosynthesis, uncoupling protein 3 (*UCP3*) induced thermogenesis, and fructose 1,6-bisphosphatase 2 (*FBP2*) changes will change the nonshivering thermogenesis of skeletal muscle (H. Li et al., 2021; Park et al., 2020). Limited by experimental conditions, there are few reports on related research on large domestic animals.

There are nearly 400 local pig breeds globally. Local breeds are generally better adapted to specific environments and examples include Tibetan pigs (Gan et al., 2019) and Piau pigs (Teixeira et al., 2021). Local breeds are important sources of genetic variability and are better adapted for production in sustainable environments. The Enshi black pig is a local breed in the central region of China. It is not obviously cold tolerant, like pigs living in more northern regions, nor is it sensitive to low temperatures like commercial pigs. Enshi black pigs are ideal for studying the low-temperature response of pigs.

Little is known about the transcriptomic responses of pigs to low temperature. In this study, we used RNA-seq analysis to determine the expression profiles of the differentially expressed genes (DEGs) responsible for cold acclimation and acute cold stress responses of Enshi black pigs. The common cold-responsive genes and pathways were then characterized. The current study provides information on the molecular mechanism of pig response to low temperature. The results may help provide strategies for breeding cold-adapted pig varieties.

1.1 | Animal materials and treatment

Enshi black pigs were provided by Huazhong Agricultural University. In October 31, 2021, nine 3-month-old female Enshi black pigs with 30 ± 3 kg body weight were selected from the pig group and randomly divided into three groups with three pigs in each group. At first, all the pigs were raised inside the pig house at a room temperature controlled at 18 ± 2°C. The outdoor temperature was 12°C/1°C (highest temperature/lowest temperature). Second, three pigs (A, cold acclimation group) were released outside and lived outside. At 55 days, three pigs (B, acute cold stress group) were moved outside. The ambient temperature at this time dropped to −17°C/−24°C (highest temperature/lowest temperature). The remaining three pigs (C, control group) remained in the temperature-controlled house. At 58 days, December 28, all of the individuals were slaughtered, and 100 mg of the longissimus dorsi muscle was taken from each and stored in liquid nitrogen. During the experiment, all individuals were guaranteed free access to food (diet components are shown in Table S1) and water. The outdoor temperature changes during the experiment are shown in Figure S1.

1.2 | RNA extraction and RNA-seq

Total RNA of the longissimus dorsi was extracted from each pig ($n = 3$ for A, $n = 3$ for B, and $n = 3$ for C) with Trizol reagent according to the manufacturer instructions. Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, USA) was used to assess the purity of the extracted RNA. Agilent 2100 RNA Nano 6000 Assay Kit (Agilent Technologies, Palo Alto, CA, USA) was used to test the concentration and integrity of total RNA. The cDNA library of all test animals was constructed and sequenced using a NovaSeq 6000 platform (Illumina, San Diego, CA, USA), and 150 bp stand-specific paired-end reads were generated. This procedure was also used to extract RNA from all test animals for use in quantitative real time–polymerase chain reaction (qRT–PCR).

1.3 | Quality control, mapping, and gene expression quantification

Raw data (raw reads) of fastq format were first processed using in-house perl scripts. In this step, clean data (clean reads) were obtained by trimming reads containing adapter, ploy-N, or low quality from the raw data. At the same time, Q20, Q30, and GC content of the clean data were calculated. All downstream analyses were performed based on clean data with high quality. Reference genome and gene model annotation files were downloaded directly from the genome website. Index of the reference genome was built using hisat2 (v2.0.5), and paired-end clean reads were mapped to the reference genome (Sus scrofa Ensembl 97 genome). STAR used the method of maximal mappable prefix (MMP), which can generate a precise mapping result for junction reads. Stringtie (v1.3.3) was used to count the read numbers mapped to each gene. FPKM was calculated, which provided the number of fragments per kilobase of transcript sequence per millions base pairs sequenced. FPKM is a simple and commonly used expression level normalization method. It normalizes both sequencing depth and genome size. Differential expression analysis of A versus C groups and B versus C groups were performed using the edgeR (3.24.3). This provided statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value < 0.05 and an absolute of $|\log_2$ fold change$| \geq 0.5$ between A versus C groups or B versus C groups were assigned as differentially expressed.

1.4 | Gene Ontology and KEGG pathway enrichment analysis

Gene Ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R package in which gene length bias was corrected. GO terms with corrected *p*-value < 0.05 were considered to be significantly enriched GO functions of the gene set. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). KOBAS was used to test the significance of enrichment of DEGs in the KEGG pathways.

1.5 | Validation of RNA-seq results by qRT−PCR

Total RNA was reverse transcribed to cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Kyoto, Japan) and stored at −20°C. Twelve genes were randomly selected for verification by qRT−PCR. The specific-primer sequences are listed in Table S2. The internal reference *β-actin* was utilized to normalize the expression data. The qRT−PCR amplification was performed under the following conditions; denaturing at 95°C for 10 min, 40 amplification cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30, followed by acquisition of fluorescence signal. qRT-PCR was performed with an SYBR Premix Ex Taq II (Takara Bio), and completed by using a LightCycler 480 II Real-Time PCR Detection System (Roche, Basel, Switzerland). The equation $2^{-\Delta\Delta Ct}$ was used to calculate the relative fold changes of RNA expression. The mean Ct values were calculated from technical triplicates.

2 | RESULTS

2.1 | Data analysis and alignment of unique reads to the pig reference genome

After the filtration of low-quality sequences and adaptor sequences, the cold adaption group, acute cold stress group, and the control group libraries produced 254, 263, and 254 million paired-end reads, respectively (Table S3). The Q20 percentages (sequencing error rates lower than 1%) were greater than 95.8%, whereas the Q30 base percentage (sequencing error rates lower than 0.1%) was greater than 89% and the GC content of each library was 51.3% on average. About 91.7% clean reads (≈707 million) were mapped to the pig reference genome using HISAT2, including 7.8% multiple mapped clean reads and 83.9% uniquely mapped clean reads (Table S4). Subsequent analysis only used the uniquely mapped clean reads.

2.2 | Identification of differentially expressed mRNAs of skeletal muscle under low temperature

A total of 45,982 mRNA were obtained and normalized with DESeq2. A volcano plot showed the expression patterns of these transcripts in the cold acclimation treatment and acute cold stress treatment. The top 10 genes were marked in the figure according to the -log10 (*p*-value) (Figure 1).

A heatmap showed the expression patterns of these transcripts in different groups and different individuals within the same group. As

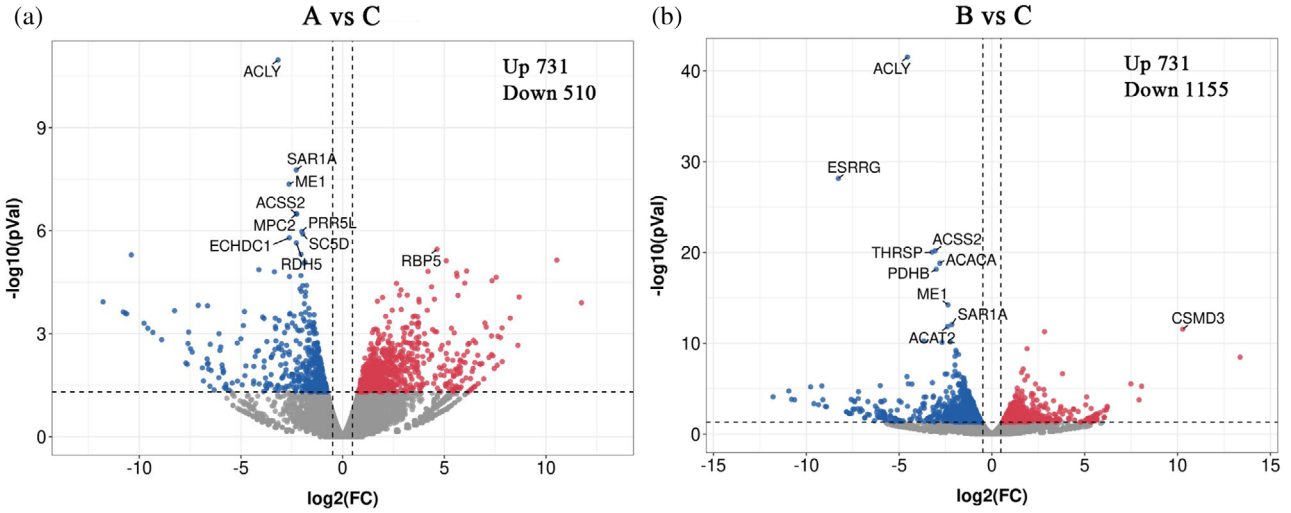

FIGURE 1 Differentially expressed mRNAs in the skeletal muscle of the Enshi black pig under low temperature. (a) Volcano plot for differentially expressed mRNAs in the cold acclimation group of Enshi black pigs. In the right of the graph, red colour is indicative of upregulated expressed genes ($\log_2$FC > 0.5 and $p < 0.05$); in the left of graph, blue colour is indicative of the downregulated genes ($\log_2$FC < −0.5 and $p < 0.05$). The grey points indicate genes that were not statistically significant ($p > 0.05$). (b) Volcano plot for differentially expressed mRNAs in the acute cold stress group of Enshi black pigs. The colour markings have the same meaning as A.

shown in Figure 2, the expression pattern of acute cold stress group and cold acclimation group were different from the control group. The differences between the acute cold stress group and cold acclimation group were not clear. In the same group, the expression pattern of the control group and acute cold stress group are basically the same, whereas in the cold acclimation group, there were large differences between individuals. This indicates that, under long cold exposure, individual responses to cold may differ.

A gene was considered differentially expressed if the $p \leq 0.05$ and the $|\log_2 FC| \geq 0.5$. Implementing this standard, a total of 1241 DEGs (including 46 unannotated) were detected in the cold acclimation group, with 731 upregulated genes and 510 downregulated genes (Table S5). A total of 1886 DEGs (including 88 unannotated) were detected in the acute cold stress group, with 731 upregulated genes and 1155 downregulated genes (Table S6). In the combined analysis, 540 genes (excluding unannotated) were common differentially expressed in the cold acclimation group and acute cold stress group, with 235 upregulated genes and 305 downregulated genes (Figure S2). Further analysis of GO and pathways enrichment involved in the cold acclimation group and acute cold stress group was carried out by these annotation DEGs. Except for the unannotated genes, there are still many annotated DEGs that remain uncharacterized. This means that some genes with unknown functions are involved in the cold response. Many molecular mechanisms of cold response remain to be discovered.

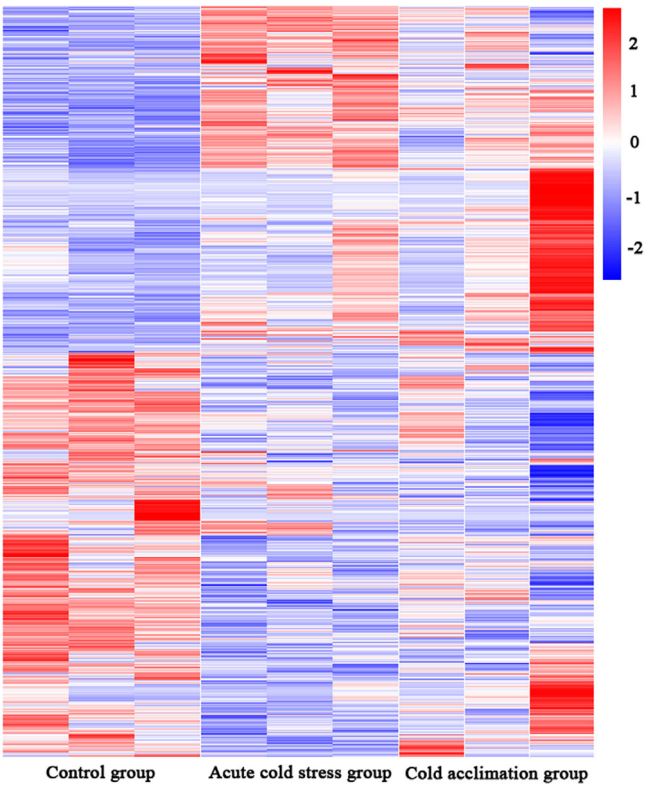

FIGURE 2 Heatmap of the different transcripts in all individuals and groups. Each column represents a sample, and each row represents one transcript. The expression level of the transcript is shown in different colours, which transition from blue to red with increasing expressions.

2.3 | GO analysis of DEGs

To analyze the potential biological function of DEGs, which respond to low temperature, the GO enrichment was displayed. All DEGs

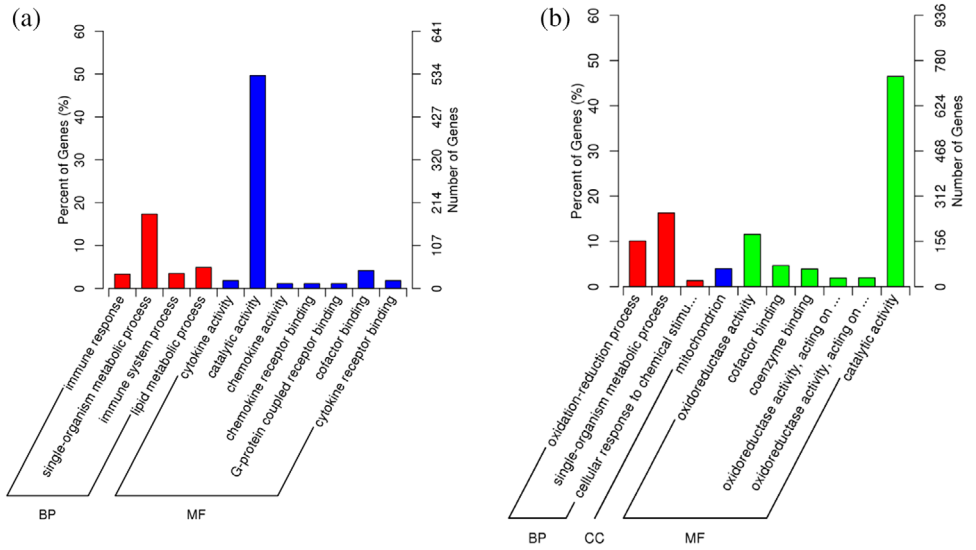

FIGURE 3 Gene Ontology (GO) analysis of differentially expressed genes (DEGs) in the cold acclimation group and the acute cold stress group. (a) GO enrichment analysis of DEGs in the cold acclimation group. (b) GO enrichment analysis of DEGs in the acute cold stress group. Abbreviations: BP, biology process; CC, cellular components; MF, molecular functions

were classified into three main GO categories: cellular components (CC), molecular functions (MF), and biological processes (BP). Although there are 1195 annotated DEGs in the cold acclimation group and 1798 annotated DEGs in the acute cold stress group, there are not many GO terms for enrichment. Under low temperature, the GO terms related to single-organism metabolic process (GO: 0044710), cofactor binding (GO: 0048037), and catalytic activity (GO:0003824) were common significantly enriched terms in the cold acclimation group and acute cold stress group. Under cold acclimation, unique GO terms related to immune response and lipid metabolic process (GO:0006955, GO:0002376, GO:0006629), cytokine activity (GO:0005125), chemokine activity (GO: 0008009), chemokine receptor binding (GO:0042379), G-protein coupled receptor binding (GO:0001664), and cytokine receptor binding (GO: 0005126) were significantly enriched (Figure 3a). Under acute cold stress, unique GO terms related to the oxidation–reduction process (GO: 0055114); cellular response to chemical stimulus (GO: 0070887); mitochondrion (0005739); oxidoreductase activity (GO: 0016491); coenzyme binding (GO: 0050662); oxidoreductase activity, acting on the CH-OH group of donors; NAD or NADP as acceptor (GO: 0016616); and oxidoreductase activity, acting on CH-OH group of donors (GO: 0016614) were enriched (Figure 3b).

2.4 | KEGG pathway analysis of DEGs

Using KEGG analysis, the DEGs involved pathway responses to low temperature that were enriched. A total of 134 pathways were enriched ($p < 0.05$) in the cold acclimation group and 98 pathways were enriched ($p < 0.05$) in the acute cold stress group. More biology pathways were affected in the cold acclimation group. A total of 71 pathways were enriched in both groups (Table S7), including the pathways of global and overview maps (2-oxocarboxylic acid metabolism, biosynthesis of amino acids, carbon metabolism, fatty acid metabolism, and metabolic pathways), lipid metabolism (unsaturated fatty acids, fatty acid, glycerolipid, and steroid), amino acid metabolism (arginine, cysteine, glycine, serine, threonine, histidine, lysine, tryptophan, tyrosine, valine, leucine, and isoleucine), carbohydrate metabolism (butanoate, citrate cycle, glycolysis/gluconeogenesis, pentose phosphate, propanoate, and pyruvate), signal transduction (AMPK, calcium, Hippo, MAPK, NF-kappa B, and TNF signalling pathway), endocrine system (adipocytokine, insulin and PPAR signalling pathway), immune system (complement and coagulation cascades, C-type lectin receptor signalling pathway, hematopoietic cell lineage, and NOD-like receptor signalling pathway), nervous system (retrograde endocannabinoid signalling and synaptic vesicle cycle), cardiovascular disease (arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy, fluid shear stress and atherosclerosis, and viral myocarditis), infectious diseases caused by bacteria or viruses (*Staphylococcus aureus* infection, human T-cell leukaemia virus 1 infection, influenza A, bacterial invasion of epithelial cells, African trypanosomiasis, and malaria), and neurodegenerative diseases (Alzheimer's disease, Huntington's disease, and Parkinson's disease).

The top 20 pathways related to DEG for cold acclimation group and acute cold stress group are shown in Figure 4a,b. According to the q-value, metabolic pathways (q = 1.60E-33), valine, leucine and isoleucine degradation (q = 6.16E-12), carbon metabolism (q = 3.70E-10), tryptophan metabolism (q = 9.59E-09), and cytokine-cytokine receptor interaction (q = 9.59E-09) were the top five pathways in the cold acclimation group. Metabolism pathways (q = 4.13E-48), Parkinson's disease (q = 2.41E-25), oxidative phosphorylation (q = 2.41E-25), Huntington's disease (q = 8.93E-22), and Alzheimer's disease (q = 3.28E-19) were the top five pathways in the acute cold stress group. Enrichment analysis revealed that the metabolic pathways were

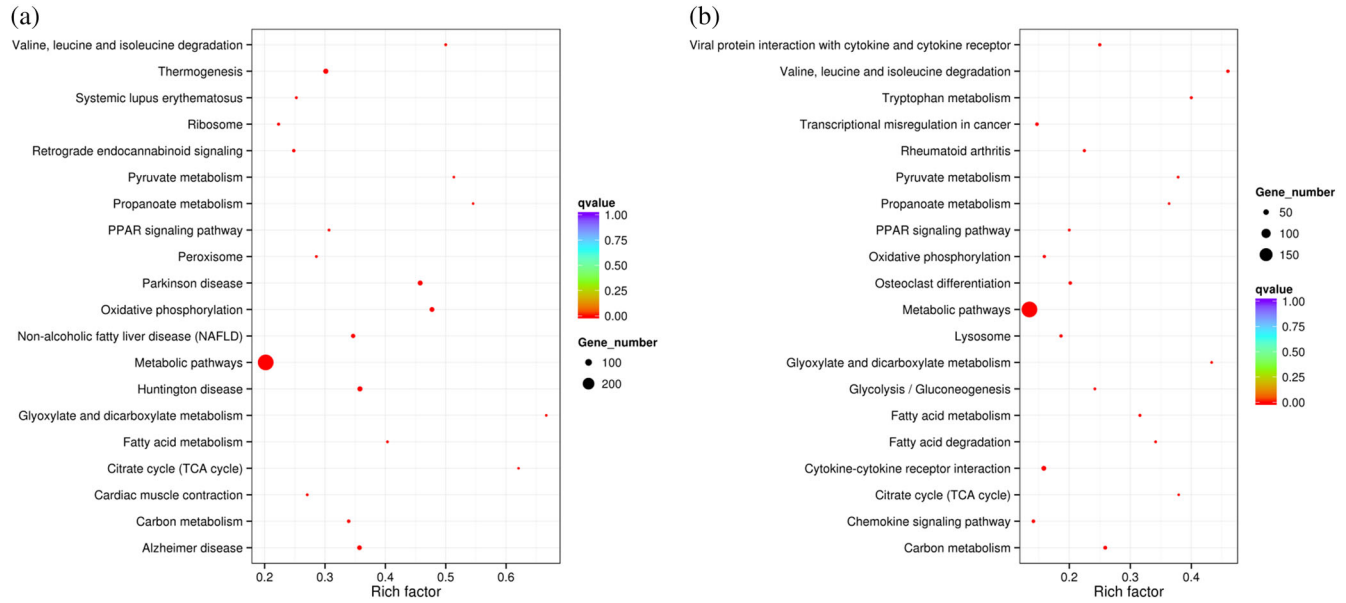

FIGURE 4 KEGG analysis of differentially expressed genes in cold acclimation group and acute cold stress group. (a) Top 20 pathways in the enrichment analysis of significant differential expressed genes ($|\log_2 FC| \geq 0.5$ and $p < 0.05$) in the cold acclimation group. (b) Top 20 pathways in the enrichment analysis of significant differential expressed genes ($|\log_2 FC| \geq 0.5$ and $p < 0.05$) in the acute cold stress group

the primary pathways among the significantly differentially expressed mRNAs for cold acclimation and acute cold stress. Under cold acclimation, the metabolism of the body is significantly affected, whereas under acute cold stress, the nervous system is significantly affected.

2.5 | Validation of DEGs

For verification of the RNA-seq results, 10 DEGs involved in the cold acclimation group and acute cold stress group were selected and verified by qRT-PCR in skeletal muscle from Enshi black pigs. The variation tendencies of DEGs were coincident with qRT−PCR. This showed that the results of RNA-seq were accurate and reliable (Figure S3).

3 | DISCUSSION

The pig is an important domestic animal that provides meat and other products. Pigs are also a suitable biomedical model for humans. Molecular mechanism research on pigs can help resolve some production problems of pig production and support human physiological research. Low temperature is a common environmental problem in animal husbandry. Response to low temperatures is a quantitative trait influenced by the interactions of many genes and the environment. Liver, adipose tissue, muscle, and brain are the main tissues that produce heat to maintain a constant body temperature upon cold exposure. Skeletal muscle is a major determinant of systemic energy homeostasis because of its large mass and high rate of fuel consumption. Some genes related to muscle fibre type conversion (Kim et al., 2021), promotion of protein processing (Tamai et al., 2022), and white adipose browning (Lee et al., 2014) play important roles in skeletal muscle heat production under cold exposure. However, RNA-seq technology, which can screen for major genes and biology pathways at the transcriptome level, has not been widely used to study the molecular mechanism of pig responses to low temperature. The molecular mechanisms of cold exposure on skeletal muscle remain unclear. To increase understanding of the skeletal muscle response of pigs to low temperature, we tested two types of low-temperature environments, including cold acclimation and acute cold stress, and analyzed common cold-responsive DEGs and pathways. Our data provide insight into the molecular responses of pigs to cold exposure.

Desaturation of fatty acids is important to maintain membrane fluidity under cold stress. With the decrease of temperature, the fluidity of the membrane will also decrease. In this study, *SCD1*, *ELOVL6*, *FADS2*, and *HSD17B4* were enriched in the biosynthesis of unsaturated fatty acids pathway, and they were all downregulated. *SCD1* is the rate-limiting enzyme catalyzing the biosynthesis of monounsaturated fatty acids. It catalyzes the synthesis of palmitoleyl-CoA and oleyl-CoA, which are the key components of membrane phospholipids and triglycerides. It was responsive to cold stress in the liver and brain of yellow croaker (H. Xu et al., 2015). *ELOVL6* is a rate-limiting enzyme catalyzing elongation of saturated and monounsaturated long-chain fatty acids. *ELOVL6* plays a role in loach adaptation to cold stress (Chen et al., 2018). *FADS2* encode delta-6 desaturase, which is an essential enzyme for the synthesis of long-chain polyunsaturated fatty acids. Intron variants in *FADS2* have been identified to be significantly associated with cold adaptation in humans (Q. Li et al., 2018). *HSD17B4* is strongly associated with phosphatidylserine (PS). PS is a negatively charged phospholipid exclusively located in the inner leaflet of the plasma membrane. In this study, *HSD17B4* was only decreased in the

acute cold stress treatment, but not changed in cold acclimation treatment. In summary, cold exposure inhibits the activity of biosynthesis of unsaturated fatty acids pathway.

The phenotype and molecular mechanism of intermuscular fat under cold exposure are unclear. High temperature enhances the accumulation of internal fat (such as perirenal fat, viscera) in pigs, but expense the external fat (such as subcutaneous fat). However, the intermuscular fat percentage is not influenced. In the present study, we found that fatty acid degradation, elongation, and glycerolipid metabolism in the skeletal muscle were slight inhibited under cold exposure. Except for the *CPT1C* gene, many genes were downregulated, including *ACADL*, *EHHADH*, *HADH*, *LIPG*, *LPL*, and *DGAT1*. Upon acute cold exposure, inguinal WAT from Misty mice compensated for BAT dysfunction by increasing expression of *ACADL* and other thermogenic genes (Motyl et al., 2013). *EHHADH* was highly expressed in gastrocnemius muscle of living high-altitude deer mice, to promote the oxidative capacity of skeletal muscle (Scott et al., 2015). *HADH* catalyzed the third reaction of the mitochondrial β-oxidation cascade, the oxidation of 3-hydroxyacyl-CoA to 3-ketoachl-CoA, for medium- and short-chain fatty acids. It is involved in thermogenesis (Schulz et al., 2011). *LIPG* encoded the endothelial lipase (EL) and can hydrolyze high-density lipoprotein (HDL). EL is a determinant of HDL lipid composition, cholesterol flux, and HDL turnover in conditions of high thermogenic activity (Schaltenberg et al., 2021). *LPL* signalling play a pivotal role in lipid metabolism (Olivecrona, 2016). Mice lacking *DGAT1*, a key enzyme in triglyceride synthesis, have increased energy expenditure and therefore are resistant to obesity. Overall, multiple genes related to fatty acid metabolism were downregulated in intermuscular fat in skeletal muscle after cold exposure, but they were induced by low temperature in adipose tissue.

Carbohydrates are a good source of fuel during strenuous environment. They can either be catabolized for energy (ATP) or used for anabolic functions. In this study, some genes enriched in carbohydrates metabolism were downregulated, such as *DLD*, *ACLY*, *ACSS2*, *FASN*, *G6PD*, and *ME1*. Some genes were upregulated, such as *PCK1*, *ACSS1*, *PGM2*, *HK3*, and *PCK1*. These take part in glycolysis to maintain carp energy requirements under cold stress. *ACSS1* plays a key role in the metabolism of acetate for energy production. The *PGM2* gene encoding phosphoglucomutase (Pgm2p) can improve galactose utilization both under aerobic and under anaerobic conditions. *HK3* is one of the isozymes of HK. HK is the first rate-limiting enzyme in cell glycolysis, and it plays an important role in regulating cell energy metabolism and cell fate. Under cold exposure, the glycolysis/gluconeogenesis pathway was activated in the skeletal muscle.

Amino acids directly trigger the synthesis of muscle proteins. Proline, tryptophan, phenylalanine, asparagine, and glutamine were all increased in the adipose tissue after cold exposure (Okamatsu-Ogura et al., 2020; Y. Xu et al., 2021). Branched-chain amino acid (BCAA; valine, leucine, and isoleucine) supplementation is often beneficial for energy expenditure. On cold exposure, BAT actively utilizes BCAA in the mitochondria for thermogenesis. In quadriceps muscle, methionine restriction upregulated lipid metabolism-associated genes and increased fatty acid oxidation, as well as stimulation of gluconeogenesis and glycogenolysis. Addition of cysteine can reverse these effects (Perrone et al., 2012). In this study, many amino acid metabolism pathways were enriched after cold exposure, including valine, leucine, isoleucine, arginine, proline, cysteine, methionine, glycine, serine, threonine, histidine, lysine, tryptophan, and tyrosine. This means that the metabolism of amino acids in skeletal muscle of pigs was affected, especially tryptophan, and many genes in this pathway were upregulated.

Cellular senescence is a stable form of cell cycle arrest that limits the proliferation of damaged cells. In this study, under low temperature, some genes were enriched in the cellular senescence pathway. *IL6*, *CXCL8*, *E2F1*, and *CCNB1* were upregulated, and *SLC25A4* and *GADD45γ* were downregulated. *IL6* and *CXCL8* are myokines that are secreted from skeletal muscle and serve a signalling role. In human myotubes, mRNA levels of *IL6* and *CXCL8* were significantly increased after cold exposure (Krapf et al., 2021). During long-term cold exposure, *IL6* plays an important role in upholding mice core body temperature through mechanisms in the central nervous system (Egecioglu et al., 2018). *E2F1* deletion leads to increased mitochondrial number and function and increased body temperature in response to cold and increased resistance to fatigue with exercise (Blanchet et al., 2012). *GADD45γ* works by activating MAPK p38, activates ERRγ independently of PGC-1 coactivators, yet synergizes with PGC-1α to induce the thermogenic program (Gantner et al., 2014). *SLC25A4* is an inner mitochondrial membrane ADP translocator. It can interact with CDKAL1 and maintain mitochondrial morphology and energy expenditure. Brief exposure to cold induces the cellular senescence that is associated with a glycolytic switch and an increase in mitochondria content (Bourdens et al., 2019).

Except for the cellular senescence pathway, focal adhesion pathway, tight junction, and peroxisomes pathways were all enriched in pigs after cold exposure. This means cellular community, transport, and catabolism were changed. But it is difficult to determine if these pathways are activated or inhabited because some genes in the pathway are always upregulated and some are downregulated. In this study, *CAV1* and *CAV2* were downregulated. They belong to the voltage-gated Ca (2+) channel family, and they serve distinct roles in cellular signal transduction. *TNC* was upregulated in the cold acclimation treatment but was not changed in the cold stress treatment. It is a part of the cardiac troponin complex and takes part in the regulation of cardiac muscle contraction. It can be induced in fish heart under cold acclimation (Genge et al., 2013). Peroxisomes are critical mediators of cellular responses to many forms of stress, including oxidative stress, hypoxia, starvation, cold exposure, and noise. Obviously, the cellular processes of skeletal muscle in pigs following cold exposure were affected, but the exact molecular mechanism remains unknown.

To survive cold temperatures, plants and animals must be able to perceive a cold signal and transduce it into downstream components that induce appropriate defence mechanisms. In this study, multiple classic signal transductions pathways were enriched, including the AMPK signalling pathway, calcium signalling pathway, ECM–receptor interaction, Hippo signalling pathway, MAPK signalling pathway, NF-kappa B signalling pathway, and TNF signalling pathway. AMPK

is a powerful potential target for metabolic diseases and energy metabolism disorders. Hypothermia and hypoxia can change the structure of rat skeletal muscle cells, enhance AMPK phosphorylation, upregulate the expression of PGC-1α, and regulate the lipid metabolism of rat skeletal muscle cells (Ruixia et al., 2021). Cold exposure induces calcium signalling pathways that promote extracellular calcium influx into cells or release intracellular calcium stores. Elevated cytosolic calcium stimulates downstream calcium-dependent signalling pathways that can also regulate muscle contraction. ECM–receptor interactions affect the differentiation of intramuscular adipocytes, and lipid synthesis and metabolism. Many studies have found that the ECM–receptor interaction pathway can improve the body cold tolerance, such as in zebrafish (Ji et al., 2020) and yaks (Lan et al., 2016). The Hippo signal transduction pathway is an important regulator of organ growth and cell differentiation. Activity of the Hippo pathway is strongly dependent on cell junctions, cellular architecture, and the mechanical properties of the microenvironment. The Hippo signalling pathway helps in the repair of vannamei intestinal structure under cold exposure. The MAPK signalling pathways control adaptive responses to intracellular and extracellular stresses, including environmental stresses and exposure to inflammatory cytokines. MAPK is the survival pathway that enhances cold resistance in zebrafish larvae (Ren et al., 2021) and is active in adolescent mice under cold exposure (B. Xu et al., 2019). The NF-kappaB pathway is a ubiquitous stress response that activates the NF-kappaB family of transcription factors. The transcriptional program that is activated is both antiapoptotic and highly proinflammatory.

Tarapacki et al. reported that, despite some differences between three cold intensities in *Drosophila suzukii*, intense and moderate cold stress induced the same physiological perturbation. They thought that cold stress caused by fluctuating conditions is additive and the rate of injury accumulation increases dramatically with decreasing temperature (Tarapacki et al., 2021). In this study, the top five pathways of the cold acclimation group mainly focus on amino acid and carbon metabolism, while the top five pathways of cold stress group mainly focus on neurodegenerative disease and oxidative phosphorylation. This means that the cold responses of the Enshi black pig to cold acclimation and cold stress are different. Analysis of all pathways, however, showed that there are more common pathways and genes in these two treatments.

4 | CONCLUSION

Low-temperature exposure will produce many DEGs, and it is difficult to explain their roles at low temperature. It is also difficult to determine the molecular mechanism behind a complex biological response such as low-temperature response by analyzing the biological pathways involved in these DEGs. The meaning of these DEGs will be elucidated in future research. We performed a comparative analysis of the transcriptome profiles of Enshi black pig skeletal muscle under conditions of cold acclimation and acute cold stress using RNA-Seq technology. Many genes and biological pathways were altered under low-temperature exposure, and we speculate that this is the molecular basis for the maintenance of body temperature in pigs at low temperatures. This is the first study of cold stress of pig skeletal muscle, and we identified many genes in this study, including some with unknown functions. These newly discovered genes may be especially important in cold environments. Our data will contribute to further studies of pig cold tolerance and provide insights into how pigs adapt to harsh environments.

AUTHOR CONTRIBUTIONS

Conceptualization: Dongjie Zhang and Di Liu. *Data curation*: Shouzheng Ma and Liang Wang. *Formal analysis*: Dongjie Zhang, Shouzheng Ma, and Liang Wang. *Funding acquisition*: Dongjie Zhang. Resources: Hong Ma. *Writing*: Dongjie Zhang and Di Liu.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

All relevant data are within the manuscript and its Supporting information files.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation, if appropriate. The experimental protocol used in this study was approved by the Animal Care and Use Committee of Heilongjiang Academy of Agricultural Sciences, People's Republic of China.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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