Transcriptome Profiling Analysis of Muscle Tissue Reveals Potential Candidate Genes Affecting Water Holding Capacity in Chinese Simmental Beef Cattle

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Research Article

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Transcriptome profiling analysis of muscle tissue reveals potential candidate genes affecting water holding capacity in Chinese Simmental beef cattle

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Abstract

Water holding capacity (WHC) is an important sensory attribute that greatly influences meat quality. However, the molecular mechanism that regulates the beef WHC remains to be elucidated. In this study, the longissimus dorsi (LD) muscles of 49 Chinese Simmental beef cattle were subjected to RNA sequencing (RNA-seq), among which eight individuals with the highest WHC (H-WHC) and the lowest WHC (L-WHC) were selected for transcriptome analysis. A total of 1256 genes were identified as differentially expressed genes (DEGs) between two groups, of which 948 genes were up-regulated and 308 genes were down-regulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment revealed that DEGs were significantly enriched in 24 GO terms and 78 pathways. Additionally, based on protein-protein interaction (PPI) network, animal QTL database (QTLdb), and relevant literature, the study not only confirmed seven genes (*HSPA12A*, *HSPA13*, *PPARγ*, *MYH10*, *MYL2*, *MYPN*, and *TPI1*) influenced WHC in accordance with previous studies, but also identified six genes (*ITGAV*, *FGF2*, *THBS1*, *DCN*, *COL4A1*, and *TGFBR1*) as the most promising novel candidate genes affecting the WHC. These findings could offer important insight for exploring the molecular mechanism underlying the WHC trait and facilitate the improvement of beef quality.

Keywords: Beef cattle; Water holding capacity; RNA sequencing; Differentially expressed genes

Introduction

Meat quality has been measured by multiple indicators such as WHC, drip loss, intramuscular fat (IMF), shear force (SF), and meat color that are economically important traits with low to medium genetic heritability (h^2) [1-5], among which WHC is an important meat sensory attribute that contributes to improving the quality and yield of meat. Previous researches about ruminants demonstrated that extremely low WHC due to myoprotein degradation was the main cause of pale, soft, and exudative (PSE) meat, while high WHC caused by high pH could explain the production of dark, firm, and dry (DFD) meat [6].

WHC is defined as a measurable characteristic related to the ability to retain inherent water in meat under the influence of intrinsic (i.e., genotype) and extrinsic (i.e., pre-slaughter and post-slaughter treatment methods) factors [7]. Drip loss is the most important method to assess WHC that defined as the fresh meat loss rate under gravity at 0-4 °C for 24 h [8]. Several studies showed that the genotype played roles in the bovine WHC trait. In the work of Martínez et al., WHC was proven to exist in differences between diversified genotypes ($p < 0.01$), which is greater in normal (+/+) bulls, intermediate in heterozygous (mh/+) bulls, and least in homozygous (mh/mh) bulls [9], which was consistent with the conclusions drawn by Uytterhaegen et al. in the Belgian Blue breed [10] and by Oliván et al. in the Asturiana de los Valles breed [11]. Age, sex, stress, and stunning during the pre-slaughter period, as well as chilling and aging in the post-slaughter period, and meat processing methods (i.e., cooking and cooling temperature, cooking and cooling rates, etc.) all influenced the WHC [7,12]. Sazili et al. suggested that in comparison with cattle stunned by low power non-penetrating mechanical stunning (LPNP) method, those stunned by high power non-penetrating mechanical stunning (HPNP) method showed a lower WHC and lightness (L*) [13]. Brad Kim et al. concluded that cryogenic freezing could lead to a significant increase in WHC and decrease SF values significantly [14]. WHC could directly affect other meat quality parameters, which was positively related to IMF content while negatively regulated drip loss and cooking loss [15-17]. PH was also a major element affecting the WHC [18]. Farouk et al. found WHC was higher in Bovine M. semimembranosus with inherently higher pH compared to lower pH [19]. Conversely, Wen et al. revealed WHC had significant and negative genetic correlations with pH[16]. The reason for the opposite conclusions of the above studies on the correlation between WHC and pH was that WHC was measured at different periods after animal slaughter.

In the researches of WHC, several candidate genes relevant to the trait have been identified in domestic animals. Serpin family G member 1 (*SERPING1*) [20], cysteine and glycine-rich protein 3 (*CSRP3*) [21], phosphorylase kinase gamma subunit (*PHKG*) [22], ryanodine receptor 1 (*RYR1*) [17], deiodinase, iodothyronine, type III (*DIO3*) [23], paired-like homeodomain 2 (*PITX2*)[24], and complement component 4 binding protein, alpha (*C4BPA*) [20] located on SSC 2, SSC 2, SSC 3, SSC 6, SSC 7, SSC 8 and SSC 9, respectively, have been proven to be related to the WHC trait of pork. Myostatin (*MSTN*) [9], peroxisome proliferator-activated receptor gamma (*PPARγ*) [25], and

myopalladin (*MYPN*) [26] mapped to BTA 2, BTA 22, and BTA 28, respectively, were identified as critical candidate genes responsible for beef WHC relying on previous studies. Besides, calpastatin (*CAST*), the specific inhibitor of the calpain family of endogenous proteases, is not only related to WHC but also correlated with tenderness in beef [27,28].

With the development of next-generation sequencing technology (NGS), high-throughput RNA sequencing (RNA-seq) has gradually become an indispensable tool for constructing transcriptome profiling and understanding the molecular mechanisms of biological processes [29]. However, few relevant studies on WHC were performed in beef and the knowledge of molecular mechanisms underlying the trait remained elusive. The WHC trait was moderate heritability (0.33 ± 0.10), showing, thereby it was not easy to improve WHC by conventional breeding methods [5]. The purpose of this study is to use the RNA-Seq technique, functional enrichment tools, PPI network, and QTLdb to identify the crucial candidate genes, significant GO terms and pathways affecting the regulation of WHC, aiming to improve the WHC trait, enhance beef quality and flourish the beef industry by using molecular breeding technologies.

Results

Phenotypic information of Chinese Simmental beef cattle

A total of 49 individuals were ranked by WHC in descending order, divided into the H-WHC group ($0.53\% \leq \text{WHC} \leq 0.70\%$; $n = 4$) and the L-WHC group ($0.30\% \leq \text{WHC} \leq 0.44\%$; $n = 4$). The average of WHC in high and low groups was 0.62% and 0.39%, respectively ($p < 0.05$). Their detailed information on carcass and meat quality traits between the two groups was presented in Table 1 and Supplementary Table S1. In comparison with the L-WHC group, the H-WHC group showed lower 35kg Water loss (%) ($p < 0.05$) of LD muscle, demonstrating WHC had a significantly negative correlation with water loss, which was consistent with previous studies [5,15,16]. Additionally, the animals grouped by WHC were similar in age and there was no significant difference in weight for pre-slaughter between them ($p > 0.05$), which could decrease the error of WHC measurement. Consequently, the samples could be used for RNA-seq to detect genes associated with the WHC.

Summary of RNA sequencing data and alignment of bovine LD muscle

The transcriptome sequencing of LD muscle tissue was conducted by RNA-seq approach for paired-end strategy (read length 150 bp) on an Illumina NovaSeq 6000 platform. As a result, a total of 186,968,565 raw reads, ranging from 19,721,321 to 29,214,147 for each sample were generated. After quality control (i.e., filtering low-quality reads), a total of 177,433,007 (an average of 22,179,126) clean reads were obtained for the 8 samples, and the quality values of Q20 and Q30 were above 98.09% and 94.37%, respectively. These results indicated that the RNA sequencing quality of the samples was high and could be used for sequence alignment. Through

alignment, an average of 97.03% of clean reads was mapped to the Bos taurus reference genome, of which 90.48-92.13% and 2.71-3.75% of clean reads per sample were uniquely mappable and multiple mappable, respectively. The information on sequencing results was listed in Table 2 and Supplementary Table S2. The alignment of clean reads confirmed the reliability of the RNA-seq, which could be used for subsequent analysis.

Transcriptome profiling of DEGs with high and low WHC

In order to investigate the transcriptome expression profiling of the LD muscle with different WHC, the gene expression levels between H-WHC and L-WHC groups were compared by using the DESeq2. Figure 1A showed two groups of individuals grouped by extreme WHC values were obviously clustered through Principal Component Analysis (PCA), which demonstrated the selection of the experimental population is reasonable. According to empirical studies, genes with a fold discovery rate (FDR) adjusted p-value less than 0.05 (padj < 0.05) and fold change ≥ 2 or fold change ≤ 0.5 ($\log_2$FC ≥ 1 or $\log_2$FC ≤ -1) were identified as DEGs. As shown in Figure 1B, compared with the L-WHC group, a total of 1256 genes were identified as DEGs in the H-WHC group, of which 948 genes were up-regulated ($\log_2$FC ≥ 1 and padj < 0.05) and 308 genes were down-regulated ($\log_2$FC ≤ -1 and padj < 0.05). The results of all DEGs were displayed in Supplementary Table S3. Furthermore, Figure 1C indicated the hierarchical clustering of heatmap depended on all DEGs was consistent with PCA analysis. Red and blue indicated the high-level and low-level gene expression in the H-WHC group versus the L-WHC group, respectively, which showed the gene expression patterns were consistent within groups and different between groups.

GO and KEGG pathway enrichment analyses

GO and KEGG enrichment analyses were performed to understand the function of the DEGs between the H-WHC and L-WHC groups. Figure 2A showed the significantly enriched GO terms (p-value < 0.01 and q-value < 0.05). A total of 24 significant GO terms were enriched, among which 15 terms were involved in biological process (BP) category (cell adhesion, biological adhesion, and muscle fiber development, etc.), eight terms were enriched in cellular component (CC) category (cell junction, extracellular matrix, and cell surface, etc.), and only one term participated in molecular function (MF) category (glycosaminoglycan binding). As shown in Table 3, among these GO terms, a large number of DEGs were enriched in cell adhesion, biological adhesion, cell surface, and extracellular matrix, implying that these biological processes might play crucial roles in the WHC trait. Figure 2B and Table 3 displayed the significantly enriched pathways of DEGs were mainly associated with environmental information processing, including the mitogen-activated protein kinase (MAPK) signaling pathway (bta04010), Calcium signaling pathway (bta04020), etc. Ten pathways were involved in human diseases and organismal systems, respectively, and two pathways were proven to be closely related to cellular processes, such as focal adhesion (bta04510) and regulation of actin cytoskeleton (bta04810). The detailed

information about significantly enriched GO and KEGG pathways was shown in Supplementary Table S4 and Supplementary Table S5. Additionally, all pathways enrichment of the DEGs were listed in Supplementary Table S6, which included the regulation of signaling molecules and interaction such as ECM-receptor interaction (bta04512) and cell adhesion molecules (bta04514). Most of the KEGG pathways were closely associated with signal transduction, cell growth, cell proliferation, cell division, cell differentiation, and muscle development.

Figure 3 showed the network diagram where the DEGs were significantly enriched in some GO terms and pathways. The DEGs associated with more than three GO terms or pathways could be recognized as potential candidate genes regulating WHC. Consequently, *ITGAV*, *FGF2*, *THBS1*, *DCN*, *COL4A1*, and *TGFBR1* were identified as novel potential candidate genes regulating WHC following the transcriptome analysis. Table 4 showed the information of these six genes.

Screening DEGs based on QTLdb and previous reports

To further search for vital candidate genes that affect WHC, we analyzed the DEGs in the cattle QTL database (https://www.animalgenome.org/cgi-bin/QTLdb/BT/index, Release 42, Aug 27, 2020). QTLs for drip loss or WHC have been found on BTA 1, 2, 4, 7, 11, 14, 19, 22, 28, and 29. However, genes influencing the WHC or drip loss identified in these QTLs remain still very limited. As listed in Supplementary Table S7, only a total of 15 QTLs in the cattle QTL database were reported to be associated with WHC and drip loss, which indicated a lack of researches on cattle WHC. Besides, we also confirmed several genes affecting the WHC that had been proven by previous studies, which was shown in Table 5. Consistent with previous studies, *HSPA12A*, *HSPA13*, *PPARγ*, *MYH10*, *MYL2*, *MYPN,* and *TPI1* were also identified as DEGs (padj < 0.05 and $|\log_2 FC| \geq 1$) in this study and these genes played an important role in the WHC trait. When broadening the threshold at only padj < 0.05, *MYPN* (padj = 3.48E-02, $\log_2 FC = 0.59$) was differently expressed in the two groups. The information on these genes could be searched in Supplementary Table S3 and Supplementary Table S8.

PPI analysis of candidate genes

To visualize the interaction between node proteins encoded by potential candidate genes, we used Search Tool for the Retrieval of Interacting Genes (STRING) for PPI network analysis, which was shown in Figure 4. Genes that had been confirmed by previous studies to be related to WHC were marked in red (*HSPA12A*, *HSPA13*, *PPARγ*, *MYH10*, *MYL2*, *MYPN,* and *TPI1*), and the potential candidate genes speculated in this experiment that influenced WHC were marked in blue (ITGAV, *FGF2*, *THBS1*, *DCN*, *COL4A1*, and *TGFBR1*). The detailed information of all candidate genes was listed in Supplementary Table S8 and the involvement of these genes in GO terms and KEGG pathways were presented in Supplementary Table S9.

Discussion

WHC is an important meat sensory attribute associated with pH, meat color, and IMF [15-17]. However, the molecular mechanisms underlying the development of WHC are still limited. In the study, we selected eight Chinese Simmental beef bulls with extremely high and low WHC to analyze their LD muscle expression profiles by using RNA-seq technology. A total of 1256 DEGs were detected, of which 948 were up-regulated and 308 were down-regulated. To identify potential candidate genes and further understand the function of critical DEGs related to WHC, GO enrichment, KEGG pathway analysis, and comparison with the QTLs influencing WHC in QTLdb and relevant literature were carried out. A total of 13 critical DEGs significantly enriched in more than three GO terms or pathways were recognized as potential candidate genes affecting WHC and the PPI network showed the relationship between key node proteins at the gene level. Our findings would provide effective information for subsequent exploration of the candidate genes in enhancing LD muscle development of cattle.

GO term and KEGG pathway analyses contribute to further understanding the structure and function of genes. In this study, DEGs were significantly enriched in 24 GO terms and 78 pathways, of which several GO terms and pathways with high-proportioned DEGs might be associated with the WHC trait. DEGs were mainly enriched in extracellular matrix (GO:0031012), collagen-containing extracellular matrix (GO:0062023), cell adhesion (GO: 0007155), and cell surface (GO:0009986). Previous studies have reported extracellular matrix (ECM) contains many proteins such as collagens, proteoglycans, and glycoproteins that affect meat quality greatly like resulting in the improvement of WHC and regulating the tenderness of meat [30-32]. In addition, ECM plays roles not only in the integrity, adaptation, and growth of skeletal muscle, but also in the adaptation of myofibrillar structures and signal transduction from the extracellular matrix to the myoblast [33]. The cell surface is composed of lipids, proteins, and carbohydrates, which regulates cell adhesion, cell-cell interactions, and communication with the environment [34]. Cell adhesion is involved in constructing the right extracellular environment and the development of skeletal muscle [35]. Integrins that belong to the superfamily of transmembrane cell adhesion proteins can bind to ECM ligands to play an important role in cell adhesion cascades [36]. Studies showed that degradation of integrins had a strong correlation with WHC [37]. Taken together, it can be speculated that ECM, cell surface, and cell adhesion may regulate the WHC trait. KEGG pathway analysis of DEGs mainly revealed that regulation of actin cytoskeleton (bta04810), focal adhesion (bta04510), ECM-receptor interaction (bta04512), and MAPK signaling pathway (bta04010) might be the crucial candidate pathway affecting WHC. The MAPK signaling pathway is an important environmental information processing that is not only involved in cell division, transcription, and translation [38], but also stimulates the growth of skeletal muscle [39]. The remolding of the actin cytoskeleton is a key part of cell processes. Zhao et al. indicated regulation of actin cytoskeleton was a potential candidate pathway affecting drip loss [40]. Huff-Lonergan et al.,

showed the changes in the architecture of myofibrils could have an impact on the ability of muscle cells to retain water, which implied the pathway of "regulation of actin cytoskeleton" involved in muscle structure perhaps was the most potential candidate pathway affecting WHC [41]. Focal adhesion is an integrin-containing and multi-protein assembly that is related to adhesion and cell signal transduction [42]. In terms of adhesion, the best-characterized aspect is muscle connection with other muscles may require an integrin-mediated linkage between the ECM and the actin cytoskeleton. All the above proves that the mentioned pathways may play special roles in the regulation of WHC.

In addition to the significantly enriched GO terms and pathways that could regulate the WHC, we revealed several candidate genes might regulate the development of WHC. Heat shock protein 70 (HSP70) was involved in WHC and tenderness due to it could protect proteins from denaturing caused by lethal heat shock [43,44]. In the work of Zhao et al., several HSP genes, *HSPA1L*, *HSPB1*, *HSPB7*, and *HSPH1*, were found to be related to drip loss [40]. In this study, heat shock protein family A (HSP70) member 13 (*HSPA13*) and heat shock protein family A (HSP70) member 12A (*HSPA12A*) were identified as DEGs between the H-WHC and L-WHC groups. HSPs are important elements of muscle that regulate the cytoskeleton and control cell maintenance [45].The improvement of these proteins abundance could contribute to less fluid exuding from the cells, thus affecting the WHC. Peroxisome proliferator-activated receptor gamma (*PPARγ*) is a ligand-activated nuclear hormone receptor subfamily of transcription factors that expresses in adipose tissue, having many functions including regulating adipogenesis, adipocyte differentiation, and glucose homeostasis [46]. Previous research showed that the mutations of the CDS region in *PPARγ* had a potential correlation with WHC and tenderness [47]. Overall, we conclude that *HSPA13*, *HSPA12A*, and *PPARγ* play an important role in beef WHC. Myopalladin (*MYPN*) is an encoding genes of the sarcomere protein that regulates Z-line and I-band protein assemblies [48]. Studies found *MYPN* regulated WHC in cattle reproduction and breeding [26]. Interestingly, Goicoechea et al., found *MYPN* was an important candidate gene for meat quality selection [49]. Although *MYPN* was differentially expressed only when padj < 0.05 in this experiment, it could also be conjectured that *MYPN* was a candidate gene that affected the WHC in accordance with previous studies. Triosephosphate isomerase (*TPI1*) was differently expressed between the two groups according to two empirical criteria (padj < 0.05 & $|\log_2 FC| \geq 1$) in this study. *TPI1* encodes triosephosphate isomerase that belongs to sarcoplasmic protein, which provides energy generation for muscle cells and is identified as a potential candidate gene related to beef meat quality like WHC [50], drip loss [51], tenderness [52], meat color [53], and ultimate pH [54]. Experiments have shown that denaturation of sarcoplasmic proteins played a special role in WHC reduction [55]. These results indicate that *TPI1* may responsible for the differences in WHC. Most of the water is stored in myofibrils [56], and the losses of water in meat are mainly owing to myofibrils swelling or shrinking [57]. Studies have indicated that denaturation of myofibrillar proteins is closely associated with low WHC [40]. Myosin

is the most abundant of myofibrillar proteins that affects the development of bovine skeletal muscles [58], which is composed of heavy (*MYH*) and light (*MYL*) chains [59]. The muscle fiber types determined by the expression of myosin heavy chain subtypes in tissues are closely related to meat quality such as WHC, drip loss, tenderness, and IMF [60,61]. In the present study, four myosin heavy chain family genes (*MYH3, MYH8, MYH10,* and *MYH11*) and two myosin light chain family genes (*MYL2* and *MYL3*) were differently expressed in the H-WHC group vs L-WHC group. Among these genes, only *MYH10* and *MYL2* were simultaneously enriched in GO terms and pathways. *MYH10*, as the muscle structural protein-coding gene, plays an important role in the kinds of muscle fiber characteristics, cytoskeleton reorganization, and focal contacts formation, which influences meat quality characteristics based on positive correlation with marbling score and negative correlation with pH and IMF [62,63]. MYL family genes have been identified as potential candidate genes for WHC prediction in the research of yak muscle with different drip loss [64]. The above shows *MYH10* and *MYL2* may be potential candidate genes regulating WHC.

In addition to the DEGs mentioned above that have been confirmed by previous researches, we also revealed several novel DEGs significantly enriched in more than three GO terms or pathways were likely to regulate the WHC. Integrin alpha-V (*ITGAV*), a member of the integrin family of extracellular matrix receptors, has been demonstrated to responsible for cell-to-matrix binding [65]. Integrins could attach the cytoskeleton to the extracellular matrix and affect the formation of drip channels [66]. Reports have shown that postmortem degradation of integrins had a positive correlation with WHC [37]. Fibroblast growth factor 2 (*FGF2*) is an integrin ligand that binds to integrin *ITGAV:ITGB3* for *FGF2* signaling, regulating skeletal myoblasts proliferation, muscle growth, cell migration, and cell survival [67,68]. Besides, thrombospondin-1 (*THBS1*) encodes the matricellular extracellular matrix adhesive glycoprotein that binds to *ITGAV* to regulate cellular processes such as cell-to-cell interactions, cell-to-matrix interactions, and focal adhesion disassembly [69]. These findings suggest *ITGAV* can interact with *FGF2* and *THBS1* to be involved in the regulation of WHC.

Collagen is an important protein in animal connective tissue that stimulates cell growth [31]. Several DEGs identified in this study are coding genes of the collagen family, including *COL4A1*, *COL5A2*, *COL6A3*, *COL8A1*, *COL12A1*, *COL15A1*, and *COL20A1* that belong to type IV, V, VI, VIII, XII, XV, XX of collagen family, respectively. Collagen alpha-1(IV) chain (*COL4A1*), as the type IV collagen proteins coding gene, has also been significantly enriched in the extracellular matrix and collagen-containing extracellular matrix terms in this study. As mentioned above, ECM contains collagens, proteoglycans, and glycoproteins that contribute to the formation of WHC [30]. Besides, some *COL4A1* mutations could influence conformational domain-containing integrin-binding sites, thus led to myopathy [70]. Consequently, its involvement in biological processes of ECM indicates that it has an effect on the WHC.

Decorin (*DCN*) belongs to a small leucine-rich proteoglycan (*SLRP*) family that is widely

distributed in the extracellular matrix to regulate skeletal muscle mass by modulating the activity of myostatin [71]. It is associated with plenty of biological functions mainly as a structural and signaling molecule, which are mediated by its interactions with cytokines, extracellular matrix proteins, and cell surface receptors [72]. *DCN* affected the rate of fibrils formation and decorin-induced signaling changes that led to increased cell migration [73]. Furthermore, studies showed that *DCN* had considerable relevance to the formation and stabilization of collagen fibers in the perimysium, which affected muscle fibers assembled with myogenesis [74]. Skeletal muscles are composed of different muscle fiber types that are closely associated with WHC and drip loss [75]. Hence, *DCN* may influence WHC by participating in the contractile and metabolic of skeletal muscle and the formation of muscle fibers.

Another well-known family of genes is the transforming growth factor, whose receptor family genes (*TGFBR1*, *TGFBR2*, *TGFBR3*, and *TGFBI*) were differentially expressed in the H-WHC and L-WHC groups. Among these genes, transforming growth factor-beta receptor 1 (*TGFBR1*) was significantly enriched in ten GO terms and nine pathways. *TGFBR1* belongs to the TGFB receptor subfamily that plays an important role in skeletal muscle development and TGF-β signal transduction [76]. Muscle fibers are the main composition of skeletal muscle, whose development is closely associated with meat quality traits in livestock such as WHC [61] and tenderness [77]. Additionally, many studies have shown that TGF-β signaling is involved in cell differentiation into myofibroblasts, ECM formation, and ECM remodeling [78]. Therefore, biological function and pathways analyses of this gene reveal that it plays a potential role in the WHC.

Conclusions

In this study, transcriptome analysis was conducted in eight individuals with extreme WHC values. A total of 948 up-regulated and 308 down-regulated genes were identified by DESeq2 in the H-WHC group versus the L-WHC group, respectively. Additionally, we revealed several GO terms (extracellular matrix, cell adhesion, cell surface, etc.), KEGG pathways (focal adhesion, regulation of actin cytoskeleton, ECM-receptor interaction, etc.), seven genes (*HSPA12A*, *HSPA13*, *PPARγ*, *MYH10*, *MYL2*, *MYPN*, and *TPI1*) confirmed by previous studies and six novel potential candidate genes (*ITGAV*, *FGF2*, *THBS1*, *DCN*, *COL4A1*, and *TGFBR1*) that may influence the WHC of beef cattle, among which the novel discovered candidate genes need further investigation and verification. These findings will provide basic and effective information for future relevant researches on beef quality traits, aiming to improve beef quality and flourish the beef industry.

Methods

Ethics declarations

The study was approved by the Ethics Committee of Science Research Department of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China (approval

number: RNL 09/07). All the animal procedures were not only performed strictly according to the guidelines proposed by the China Council on Animal Care and the Ministry of Agriculture People's Republic of China, but also in compliance with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines. The use of anilmals and private land in this study was approved by their respective legal owners.

Animals and sample collection

A total of 49 Chinese Simmental beef bulls with an average age of 26 months and an average pre-slaughter weight of 700kg were obtained to eliminate the influence of farm, age, and sex differences on the results of the longissimus dorsi (LD) muscle transcriptome. These cattle were from Inner Mongolia Aokesi Livestock Breeding Co., Ltd and were raised in the same feeding strategies and conditions. Slaughtering and sampling were completed in Zhongao Food Co., Ltd (Aohan Banner, Chifeng City, Inner Mongolia). Cattle stopped feeding and drinking strictly 24 hours before slaughter. The longissimus dorsi (LD) muscle (12-13th ribs) was harvested within 30 min after slaughter and the samples were washed with phosphate-buffered saline (PBS) to avoid contaminating the muscle tissues during the operation. Afterward, pieces of LD muscle tissues were obtained and put into Eppendorf (EP) tubes. All samples were immediately frozen in liquid nitrogen for total RNA extraction. In addition, 1 kg of the LD muscle (11-13th ribs) of the left carcass per sample was collected at 24 h after slaughter to measure meat traits including WHC and the rate of 35kg water loss using TA-XT plus Texture Analyser 12785 (Stable Micro Systems Ltd, Godalming, Surrey GU7 1YL, UK) according to reference NY/T 1333-2007.

Total RNA extraction, library construction, and sequencing

Total RNA was isolated from individual LD tissue using TRIzol reagent (Invitrogen, Life Technologies) according to the protocol of instruction. The concentration, purity, and integrity of RNA were used to evaluate the total RNA quality. The RNA concentration was tested by Qubit® RNA Assay Kit(Life Technologies, CA, USA), RNA purity was assessed using NanoPhotometer® spectrophotometer(Thermo Fisher Scientific, MA, USA), and RNA integrity was measured through the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then, high-quality samples (28S/18S > 1.8 and OD 260/280 ratio > 1.9) were used to construct cDNA libraries and applied for RNA sequencing if the RNA Integrity Number (RIN) was more than 7. The construction of cDNA libraries was generated using IlluminaTruSeqTM RNA Kit(Illumina, USA) following the manufacturer's instructions and the RNA sequencing was performed on an Illumina NovaSeq 6000 platform by paired-end strategy (read length 150 bp). The RNA sequencing was completed by Beijing Novogene Technology Co., Ltd.

Quality control of sequencing data

To obtain clean reads, the MD5 value was used to check the integrity of the original sequencing

read. Using FastQC (v0.11.9) to evaluate the read quality in terms of base composition and quality distribution, then visualizing all sequencing results through MultiQC(v1.9). Using adaptive trimming algorithm of Trimmomatic (v0.39) tools to perform quality filtering, discarding reads containing ploy-N (the percentage of undetermined base information is greater than 5% in a read), trimming adaptors and low-quality reads. Subsequent data analysis is based on clean reads obtained through the above steps.

Reads mapping

HISAT2 (v2.2.1) was used to compare clean reads to reference genome Bos taurus ARS-UCD1.2 (ftp://ftp.ensembl.org/pub/release-101/fasta/bos_taurus/dna/) [79]. Effective reads aligned to the gene region were statistically calculated according to the genomic location information specified by the cattle reference genome annotation (ftp://ftp.ensembl.org/pub/release-101/gtf/bos_taurus/). SAM files generated by the HISAT2 were sorted through SAMtools (v1.11). FeatureCounts (v1.5.2) was used to estimate read counts generated from RNA sequencing experiments [80].

Differentially expressed genes identification and function enrichment analysis

All the cattle were sorted in descending order of the WHC value and eight individuals with significant differences in the WHC were selected for analyzing their transcriptome differences to identify potential candidate genes affecting the WHC. Differential gene expression analysis was analyzed using DESeq2 (v1.18.0) [81], which provides statistical routines for calculating differential expression based on the negative binomial distribution. Benjamini-Hochberg approach was used to adjust P-values for controlling the false discovery rate (FDR). Genes with padj < 0.05 and $\log_2FC \geq 1$ or $\log_2FC \leq -1$ were identified as DEGs. Heatmap was drawn by pheatmap (v1.1.7) package [82]. To understand the function of DEGs, GO and KEGG pathway enrichment analyses were performed using the R package "clusterProfiler" based on the hypergeometric model [83]. GO term analysis was divided into three categories, namely, biological process (BP), cellular component (CC), and molecular function(MF). KEGG pathway analysis revealed the role of DEGs in metabolic pathways and specific biological functions. Those GO terms and pathways showing an adjusted p-value of less than 0.01 and q-value less than 0.05 for each term were considered to be significantly enriched. We further used the Search Tool for the Retrieval of Interacting Genes (STRING) to carry out protein-protein interaction (PPI) network analysis.

DEGs comparison with the QTLs and previous reports affecting WHC

With the development of high-throughput sequencing technologies, the genetic mapping of quantitative trait loci (QTL) has provided well-defined genetic maps for meat quality traits [84]. The Animal Quantitative Trait Loci (QTL) Database (Animal QTLdb) is open and free of charge that provides dynamic, updated publicly available trait mapping data to locate and compare discoveries within and between species. Up to now, a total of 160,659 QTLs/associations from 1,030

publications that contain 675 phenotypic traits have been collected in the current release of the Cattle QTL database (QTLdb, https://www.animalgenome.org/cgi-bin/QTLdb/BT/index). In order to screen the DEGs for the candidate genes associated with beef WHC, we compared the DEGs with QTLs in the cattle QTLdb and previous reports of WHC trait. The DEGs mapping to QTL related to the WHC trait deserved further investigation and discussion.

Statistical analysis of animal performance

Using the Independent-Sample T-test procedure of SPSS (v20.0) to assess the measurement results of meat traits. All data presented in the table were expressed as means ± standard deviation (M ± SD). Meat quality evaluation by reference NY/T 1333-2007.

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Author contributions statement

H.J.G. and J.Y.L. designed and supervised the experiments. L.L.D. and T.P.C. performed the experiments and drafted the manuscript. B.X.A., M.L., and X.H.D. analyzed the data. W.T.C., B.Z., X.G., Y.C., and L.P.Z. helped to conduct the study. All authors have read and approved the final manuscript.

Availability materials

The following are available at supplementary materials, Supplementary Table S1 Phenotypic information of WHC and other traits for the low and high samples, Supplementary Table S2 The primary information of sequencing reads alignments to Bos taurus reference genome, Supplementary Table S3 All DEGs detected between high and low WHC groups, Supplementary Table S4 GO terms significantly enriched with DEGs , Tsupplementary able S5 Top 20 pathways enriched based on the number of DEGs enriched per pathway , Supplementary Table S6 All KEGG pathways significantly enriched with DEGs , Supplementary Table S7 The detailed information candidate genes affecting WHC trait, Supplementary Table S8 The information of potential candidate genes involved in GO terms and pathways, Supplementary Table S9 Comparison of DEGs with QTLdb and previous reports influencing WHC or drip loss.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Figure legend

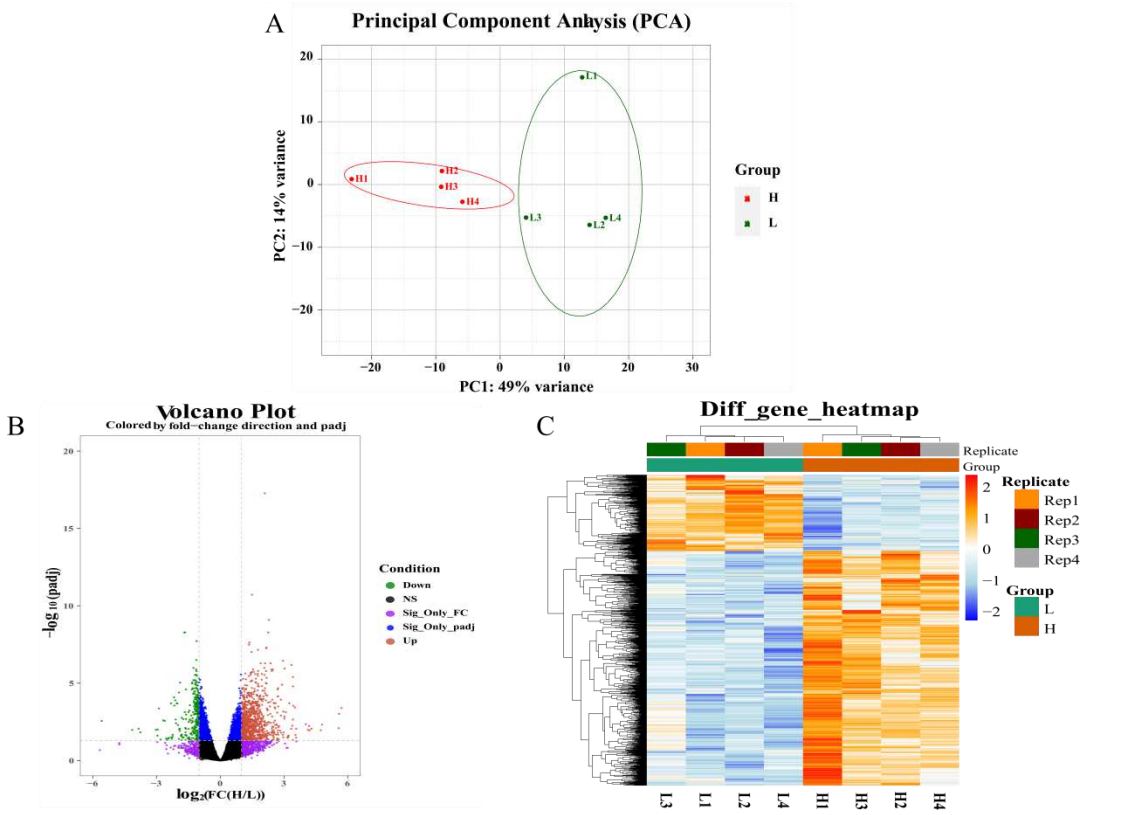
Figure 1. Samples correlation analysis and identification of DEGs between high WHC and low WHC groups. (A) PCA of the identified genes. The red and green dots represent samples of high WHC and low WHC, respectively. The high WHC and low WHC samples were obviously clustered. (B) Volcano plot for DEGs in LD muscle comparing high WHC group versus low WHC group. The red and green dots represent significant up-regulated (FC ≥ 2 and padj ≤ 0.05) and down-regulated (FC ≤ 0.5 and padj ≤ 0.05) DEGs, respectively. Dots of other colors indicate genes that are not significant. The purple dots denote genes with FC ≥ 2 or FC ≤ 0.5 and padj > 0.05, while the blue dots indicate genes only meet the condition of padj ≤ 0.05. The black dots represent genes with no significant change (0.5 < FC < 2 and padj > 0.05). (C) Heatmap of DEGs. Columns and rows show samples and DEGs, respectively. Red indicates high level gene expression in H-WHC versus L-WHC group, while blue represents low level gene expression in H-WHC versus L-WHC group.

Figure 2. GO terms and KEGG pathways analyses of all DEGs between H-WHC and L-WHC groups. The x-axis and y-axis represent the number of DEGs enriched per GO term or KEGG pathway, and the most highly enriched GO terms or pathways, respectively. Three GO categories (BP, CC, and MF) and four KEGG A_Class are shown in different colors. The numbers in the figure represent the number of DEGs enriched to each GO term or pathway.

Figure 3. The network diagram of the most critical DEGs and their belonged GO terms and pathways. Blue, purple, and orange squares represent the enriched GO terms. Green squares represent the enriched pathways. Gene marked in blue displays the critical DEGs that may be candidate genes affecting the WHC.

Figure 4. PPI network of the critical candidate genes affecting the WHC. Genes marked in red represent they have been confirmed by predecessors to be related to WHC, while the potential candidate genes influencing WHC found in this experiment are marked in blue.

1


A
Principal Component Analysis (PCA)
PC2: 14% variance
PC1: 49% variance
Group
H
L
B
Volcano Plot
Colored by fold−change direction and padj
−log 10 (padj)
log2(FC(H/L))
Condition
Down
NS
Sig_Only_FC
Sig_Only_padj
Up
C
Diff_gene_heatmap
Replicate
Group
Rep1
Rep2
Rep3
Rep4
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H
L3
L1
L2
L4
H1
H3
H2
H4

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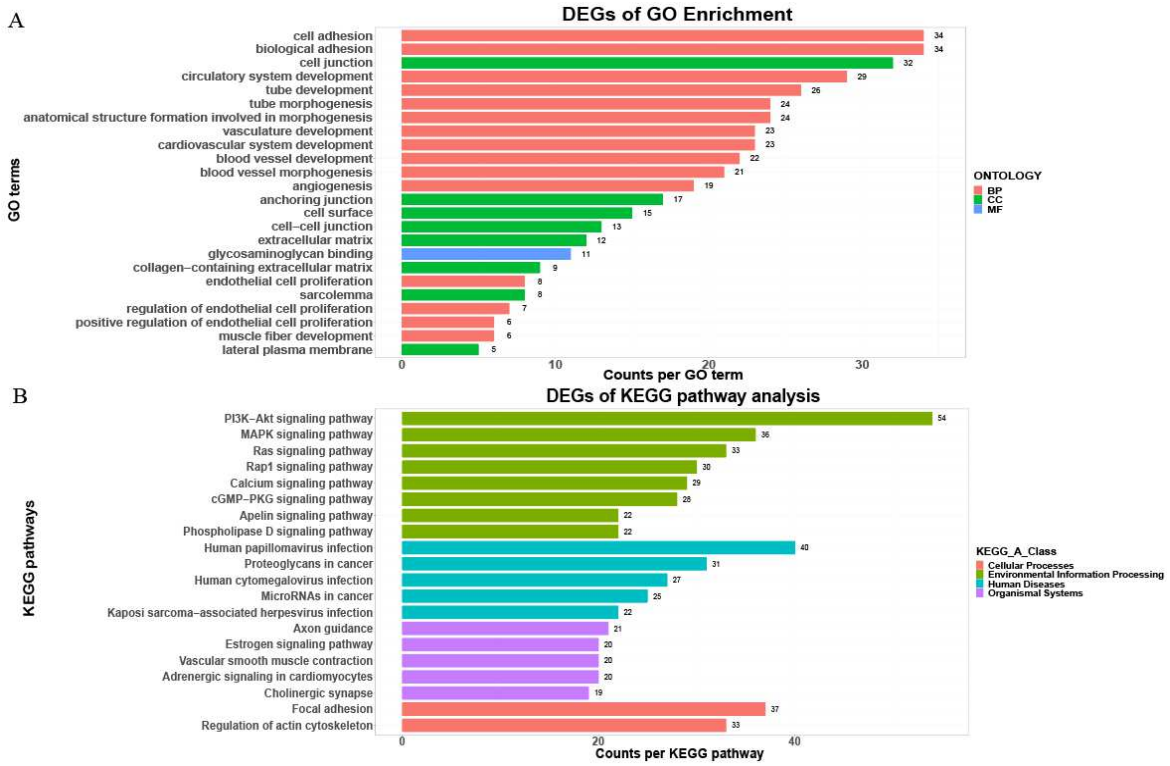
A
DEGs of GO Enrichment
GO terms
cell adhesion
biological adhesion
cell junction
circulatory system development
tube development
tube morphogenesis
anatomical structure formation involved in morphogenesis
vasculature development
cardiovascular system development
blood vessel development
blood vessel morphogenesis
angiogenesis
anchoring junction
cell surface
cell−cell junction
extracellular matrix
glycosaminoglycan binding
collagen−containing extracellular matrix
endothelial cell proliferation
sarcolemma
regulation of endothelial cell proliferation
positive regulation of endothelial cell proliferation
muscle fiber development
lateral plasma membrane
Counts per GO term
ONTOLOGY
BP
CC
MF
B
DEGs of KEGG pathway analysis
KEGG pathways
PI3K−Akt signaling pathway
MAPK signaling pathway
Ras signaling pathway
Rap1 signaling pathway
Calcium signaling pathway
cGMP−PKG signaling pathway
Apelin signaling pathway
Phospholipase D signaling pathway
Human papillomavirus infection
Proteoglycans in cancer
Human cytomegalovirus infection
MicroRNAs in cancer
Kaposi sarcoma−associated herpesvirus infection
Axon guidance
Estrogen signaling pathway
Vascular smooth muscle contraction
Adrenergic signaling in cardiomyocytes
Cholinergic synapse
Focal adhesion
Regulation of actin cytoskeleton
Counts per KEGG pathway
KEGG_A_Class
Cellular Processes
Environmental Information Processing
Human Diseases
Organismal Systems

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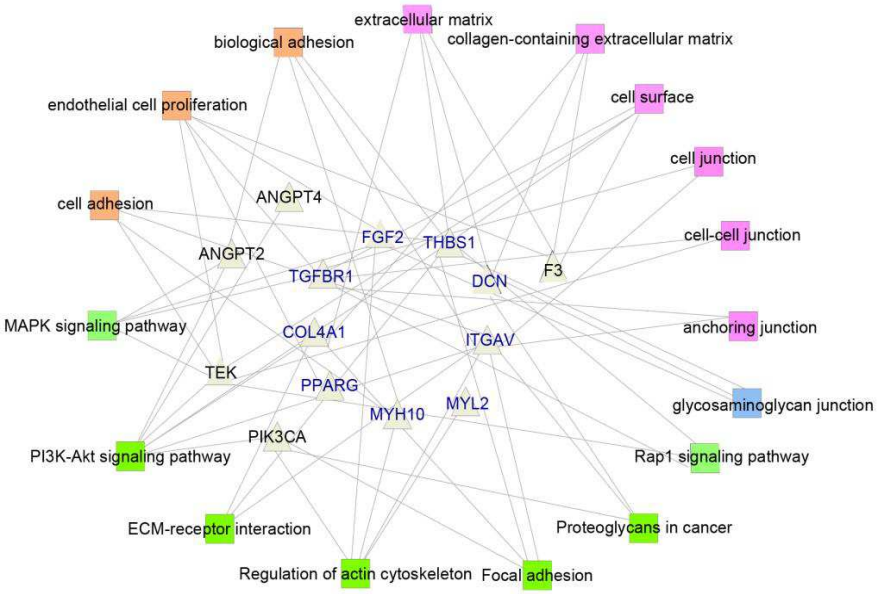

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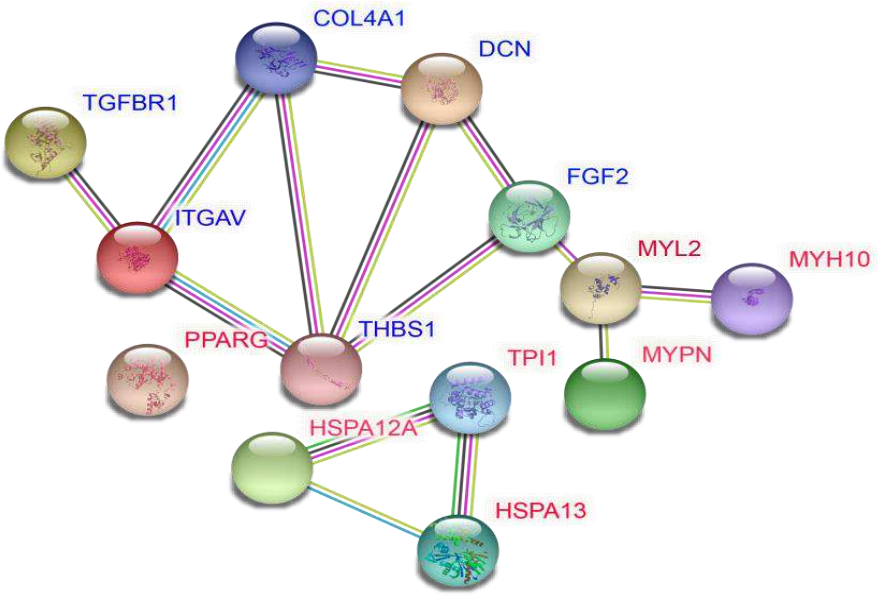

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820 **Tables**

821 **Table 1.** Summary of statistical data for WHC and other traits between samples with high WHC (n
822 = 4) and low WHC (n = 4), respectively.

Characters	All samples measured	High WHC group	Low WHC group	p
WHC(%)	0.50 ± 0.08	0.62 ± 0.09	0.39 ± 0.07	0.009
35kg Water loss (%)	0.36 ± 0.06	0.28 ± 0.07	0.45 ± 0.05	0.007
Weight for pre-slaughter (kg)	702.5 ± 78.49	751.0 ± 69.06	668.3 ± 92.69	0.206

823 p = p-value calculated by Independent-Sample T-test procedure of SPSS (v20.0)
824 The results are shown by mean±standard deviation
825
826 **Table 2.** Summary of sequencing reads alignments to the Bos taurus reference genome

Sample	Clean reads	Total mapped reads(%)	Uniquely mapped reads(%)	Multiple mapped reads(%)
H1	21,688,061	97.32	92.13	2.71
H2	23,789,046	97.23	91.52	3.21
H3	19,721,321	96.98	91.07	3.31
H4	21,220,022	96.86	90.98	3.18
L1	29,214,147	96.93	90.98	3.75
L2	26,307,213	96.87	90.48	3.71
L3	20,406,566	96.86	90.75	3.58
L4	24,622,189	97.16	91.79	3.28

827 H1, H2, H3, and H4 represent four samples of the highest WHC group; L1, L2, L3, and L4
828 represent four samples of the lowest WHC groups.
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847 **Table 3.** Most important GO terms and pathways of DEGs between H-WHC and L-WHC groups

Classification	GO term/pathway	p-value	Number of genes	Key genes
GO terms	cell adhesion	1.41E-07	34 (3)	*ITGAV/THBS1/MYH10*
	biological adhesion	2.14E-07	34 (3)	*ITGAV/THBS1/MYH10*
	endothelial cell proliferation	2.77E-04	8 (3)	*TGFBR1/FGF2/PPARγ*
	cell junction	1.20E-03	32 (2)	*ITGAV/TGFBR1*
	anchoring junction	1.06E-04	17 (2)	*ITGAV/TGFBR1*
	cell surface	1.49E-04	15 (2)	*TGFBR1/THBS1*
	extracellular matrix	4.12E-05	12 (3)	*DCN/THBS1/COL4A1*
	collagen-containing extracellular matrix	7.06E-05	9 (2)	*DCN/COL4A1*
Pathways	MAPK signaling pathway	9.66E-07	36 (5)	*TEK/TGFBR1/TGFBR2/FGF2/ FGFR1*
	Focal adhesion	8.76E-12	37 (3)	*ITGAV/ITGA6/COL6A3*
	Regulation of actin cytoskeleton	1.92E-08	33 (2)	*ITGAV/ITGA6*
	ECM-receptor interaction	2.45E-06	17 (7)	*ITGAV/IITGA6/ITGA8/ITGA9/ COL4A1/COL6A3/THBS1*
	Cell adhesion molecules	4.53E-03	17 (4)	*ITGAV/ITGA6/ ITGA8/ ITGA9*

848 GO: Gene Ontology
849 KEGG: Kyoto Encyclopedia of Genes and Genomes
850 DEGs: differently expressed genes
851 MAPK: mitogen-activated protein kinase
852 ECM: extracellular matrix
853 Number of genes: the first number represent the total number of genes enriched in per GO term or
854 pathway; the second number represents the number of key genes displayed in the next column.
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Table 4. Six potential candidate genes affecting the WHC between two groups

Symbol	BTA	log_2FC	padj	Gene position (bp)	Gene description
ITGAV	2	1.47	5.83E-05	9644368-9749556	integrin subunit alpha V
FGF2	17	1.60	5.31E-04	34801330-34860849	fibroblast growth factor 2
THBS1	10	2.28	8.21E-10	35209595-35224867	thrombospondin 1
DCN	5	1.22	2.77E-03	21014376-21053400	decorin
COL4A1	12	1.12	2.13E-02	84863917-84995777	collagen type IV alpha 1 chain
TGFBR1	8	1.23	3.12E-03	64107418-64179245	transforming growth factor beta receptor 1

WHC: water holding capacity

BTA: Bos taurus Autosome

FC: fold change

padj: p-value adjusted by false discovery rate (FDR)

Gene position (bp): position (bp) on ARS-UCD1.2

Table 5. Previous reports related to WHC or drip loss

Symbol	BTA	Gene position(bp)	GC content(%)	Reference
HSPA1L	23	27523225-27527209	45.55	Reference [40]
HSPB1	25	34345339-34347009	67.44	Reference [40]
HSPB7	2	136053155-136057934	64.67	Reference [40]
HSPH1	12	29796159-29819628	39.42	Reference [40]
PPARγ	22	56709248-56835386	41.26	Reference [25,47]
MYH1	19	29483027-29507056	41.84	Reference [61]
MYH7	10	21325414-21345624	55.05	Reference [85]
MYH10	19	28063029-28183409	44.00	Reference [61]
MYL2	17	54706765-54714580	44.93	Reference [64]
MYPN	28	24679611-24593260	39.69	Reference [26]
MSTN	2	3631373-3851228	34.05	Reference [9]
TPI1	5	103580087-103583951	60.26	Reference [50]

BTA: Bos taurus Autosome

Gene position (bp): position (bp) on ARS-UCD1.2

Figures

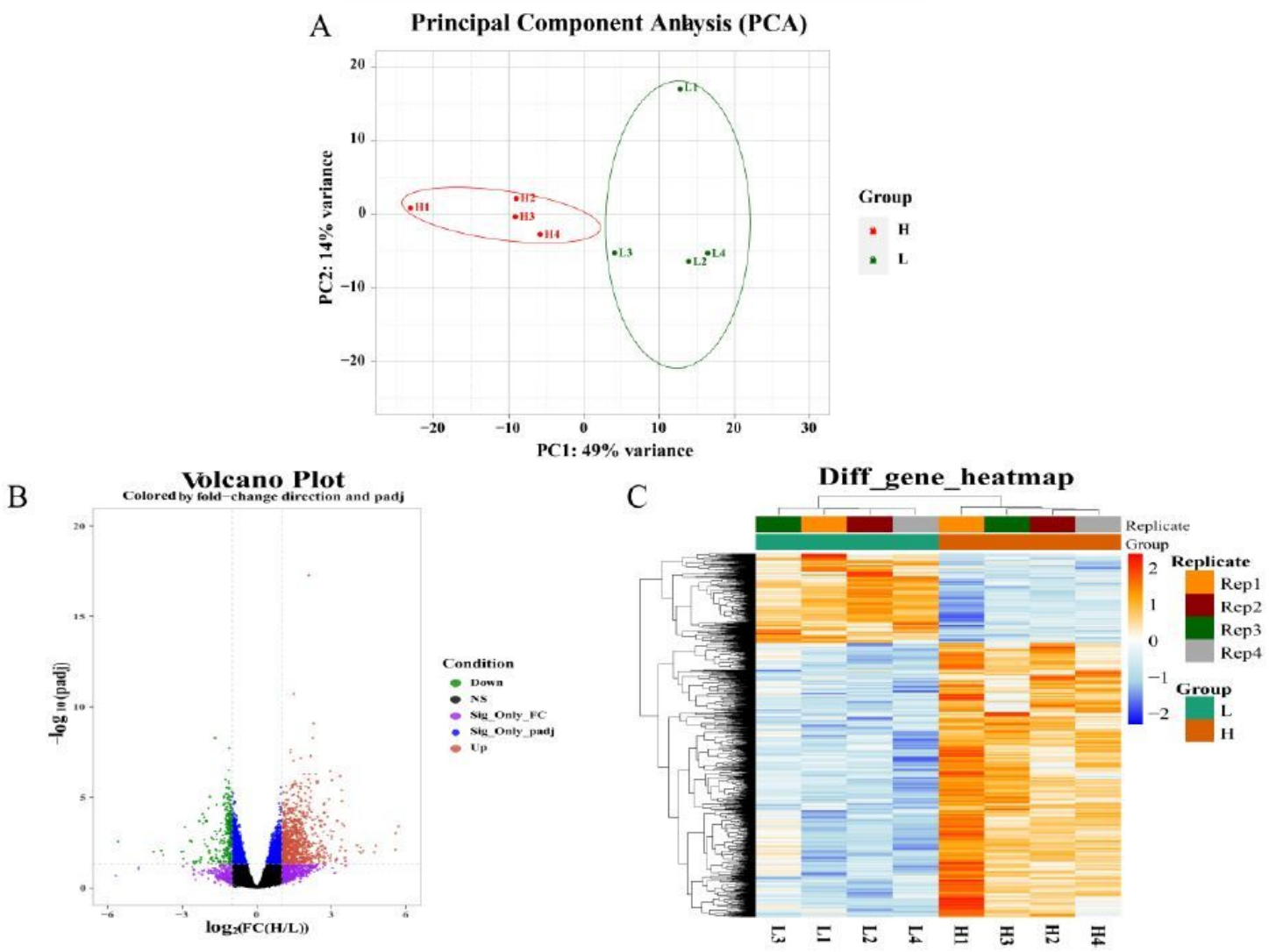

Figure 1

"See the Supplemental Files section for the complete figure caption".

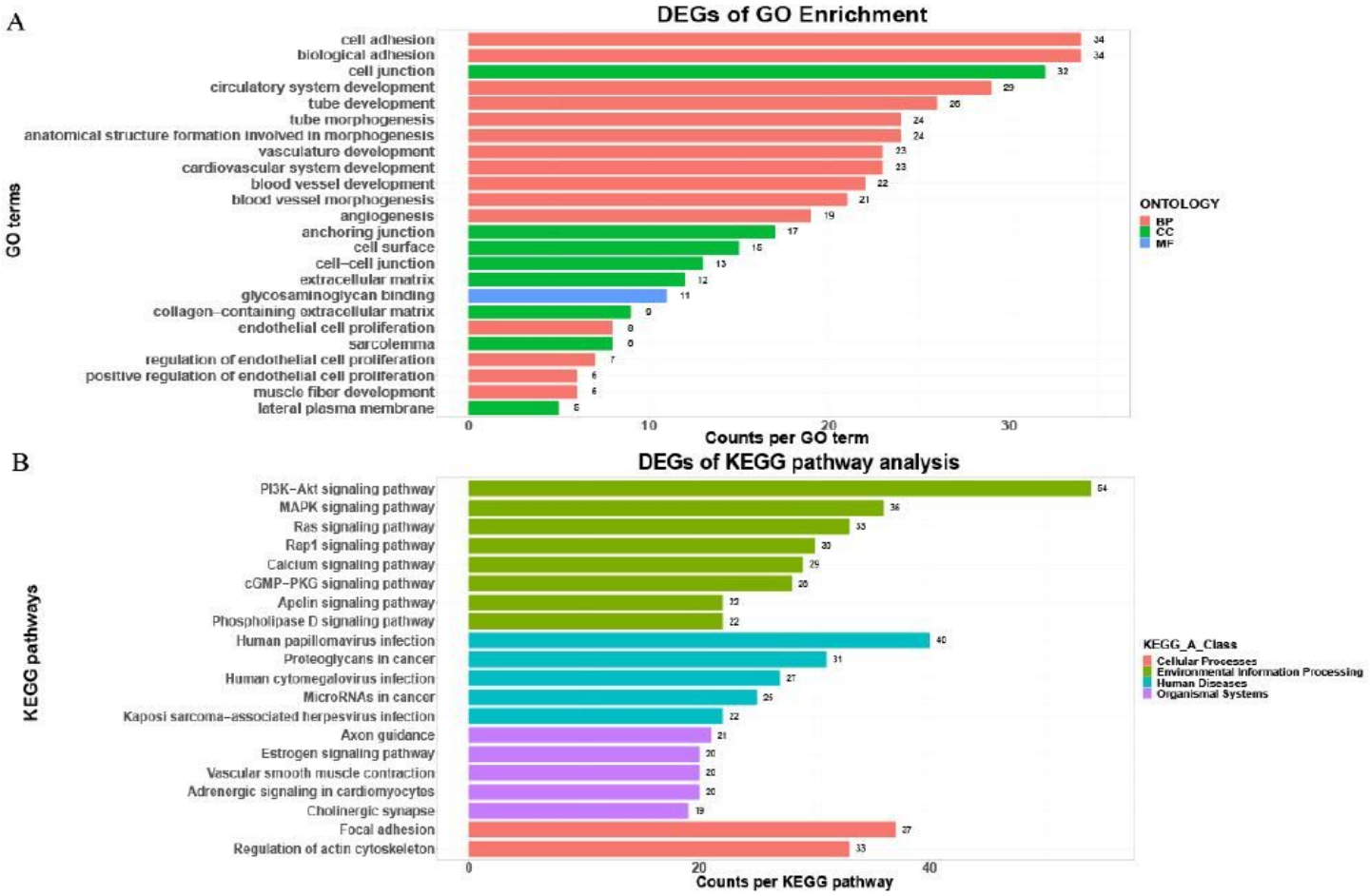

Figure 2

"See the Supplemental Files section for the complete figure caption".

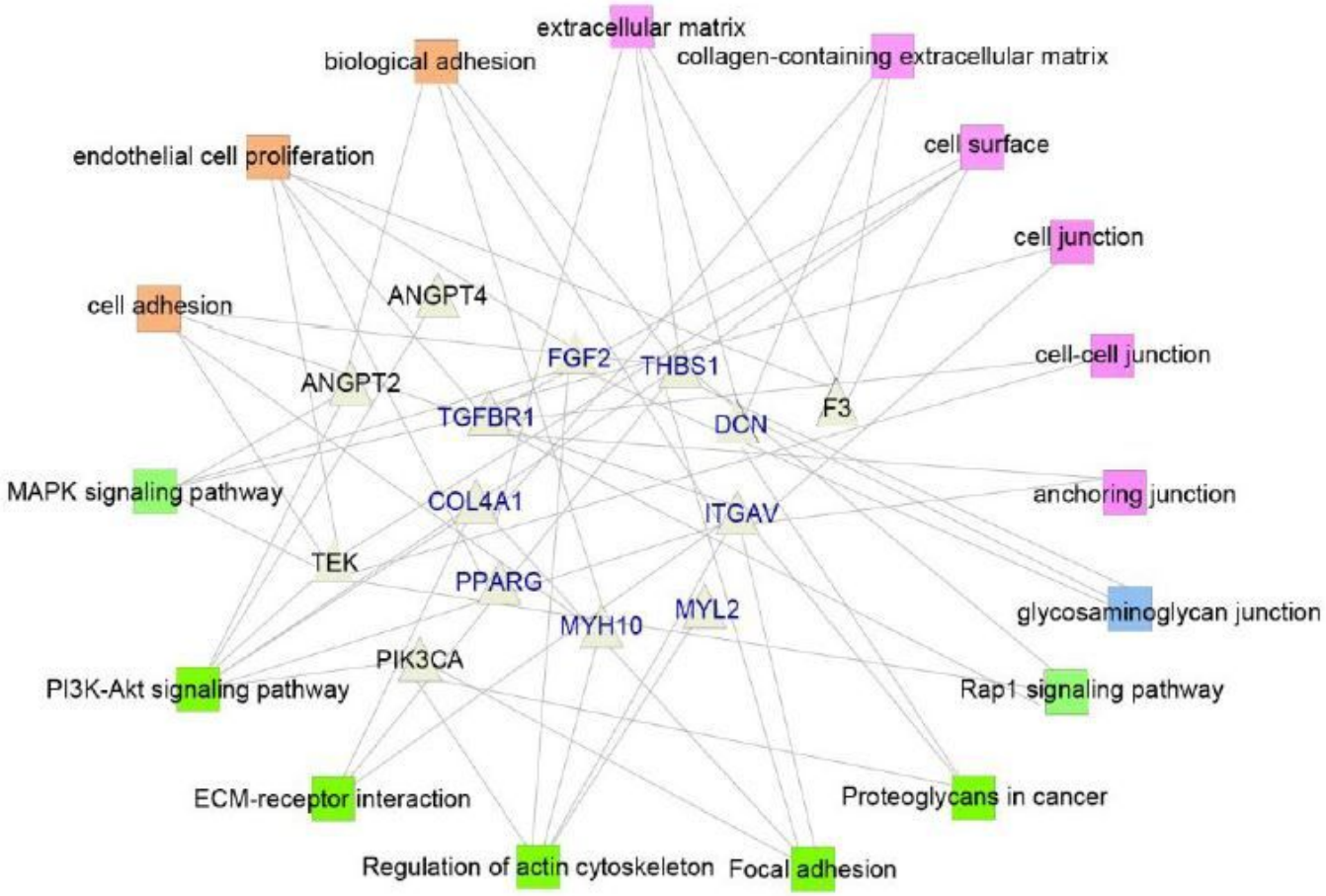

Figure 3

"See the Supplemental Files section for the complete figure caption".

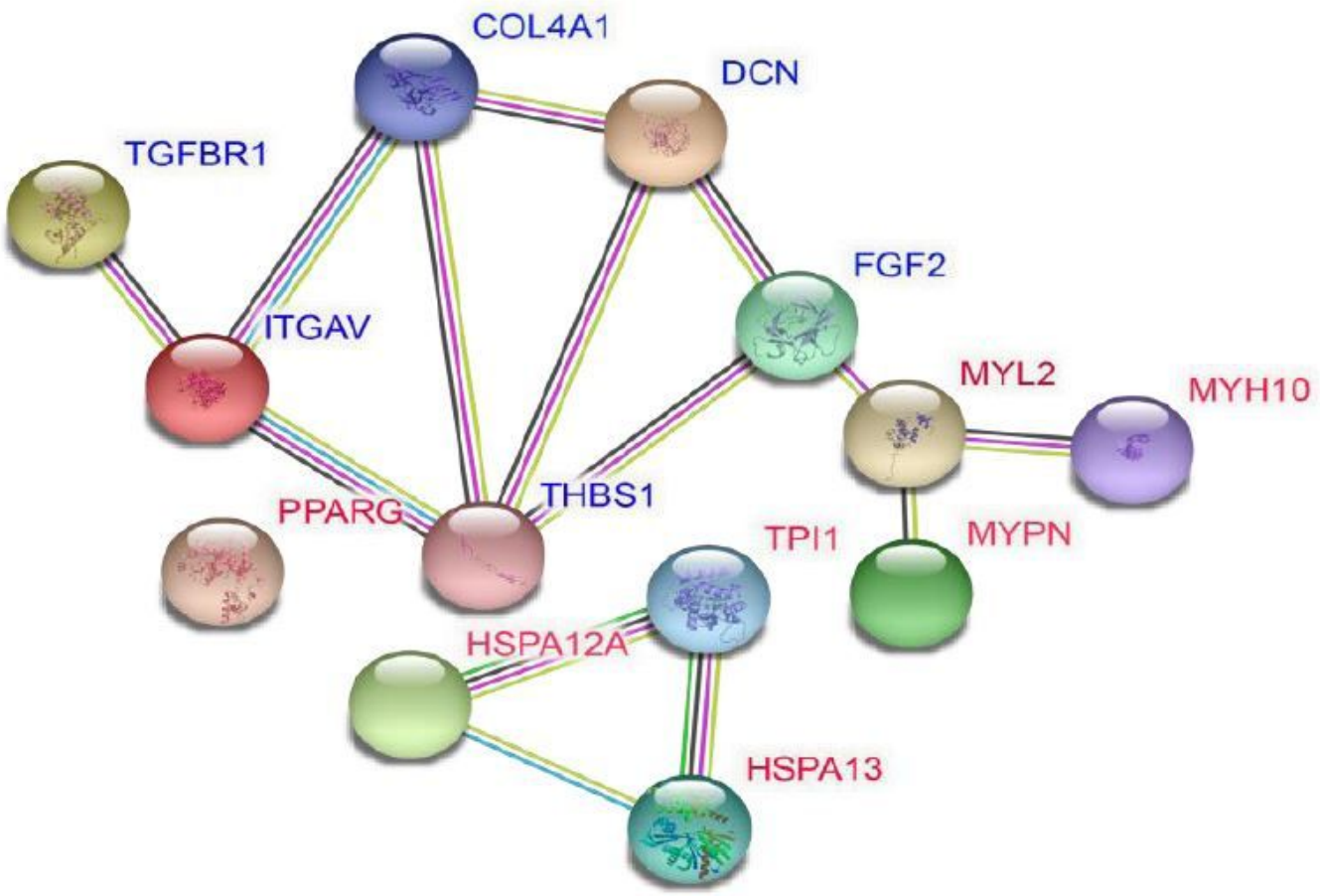

Figure 4

"See the Supplemental Files section for the complete figure caption".

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1PhenotypicinformationofWHCandothertraitsforthelowandhighsamples.xlsx
- TableS2Theprimaryinformationofsequencingreadsalignmentstobostaurusreferencegenome.xlsx
- TableS3AllDEGsdetectedbetweenhighandlowWHCgroups.xlsx
- TableS4GOtermssignificantlyenrichedwithDEGs.xlsx
- TableS5Top20pathwaysenrichedbasedonthenumberofDEGsenrichedinperpathway.xlsx
- TableS6AllKEGGpathwayssignificantlyenrichedwithDEGs.xlsx
- TableS7ComparisonofDEGswithQTLsinfluencingWHC.xlsx
- TableS8Thedetailedinformationcandidategenesaffectingwhctrait.xlsx

- FigureCaptions.pdf
- TableS9TheinformationofcandidategenesinvolvedinsomesignificantGOtermsandKEGGpathways.xlsx