Comparative analysis of differentially abundant proteins between high and low intramuscular fat content groups in donkeys

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Intramuscular fat (IMF) is an important regulator that determines meat quality, and its content is closely related to flavor, tenderness, and juiciness. Many studies have used quantitative proteomic analysis to identify proteins associated with meat quality traits in livestock, however, the potential candidate proteins that influence IMF in donkey muscle are not fully understood. In this study, we performed quantitative proteomic analysis, with tandem-mass-tagged (TMT) labeling, with samples from the longissimus dorsi (LD) muscle of the donkey. A total of 585,555 spectra were identified from the six muscle samples used in this study. In total, 20,583 peptides were detected, including 15,279 unique peptides, and 2,540 proteins were identified. We analyzed differentially abundant proteins (DAPs) between LD muscles of donkeys with high (H) and low (L) IMF content. We identified 30 DAPs between the H and L IMF content groups, of which 17 were upregulated and 13 downregulated in the H IMF group. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis of these DAPs revealed many GO terms (e.g., bone morphogenetic protein (BMP) receptor binding) and pathways (e.g., Wnt signaling pathway and Hippo signaling pathway) involved in lipid metabolism and adipogenesis. The construction of protein–protein interaction networks identified 16 DAPs involved in these networks. Our data provide a basis for future investigations into candidate proteins involved in IMF deposition and potential new approaches to improve meat quality in the donkey.

KEYWORDS
donkey, IMF, proteomics, differentially abundant proteins, functional analysis
Introduction

Donkeys are domesticated animals that belong to the horse family (1). They are one of the most important livestock animals in many countries in Africa and the Middle East, which are mainly used for farming or other work on large farms (2, 3). With the increase of interest in donkey breeding, it is increasingly being used as a milk and meat-producing animal (4, 5). Donkey milk has been shown to be the best substitute for human milk for children who are allergic to milk proteins (6). Donkey meat is characterized by high-quality protein, vitamins, and minerals, which is the preferred meat for many consumers due to its high protein content (5, 7, 8). According to a previous study, 100 g of donkey meat contains 22.8 g of protein and 2.02 g of fat (2, 9, 10). With improvements in living standards, consumers are becoming more concerned about the quality of livestock meat. In recent years, donkey meat consumption has increased in many countries, including China and Italy, and has undoubtedly become one of the livestock meat choices (6, 11, 12).

Intramuscular fat (IMF), also referred to as marbling, is the amount of fat that accumulates between muscle fibers or inside muscle cells (13). Its main components are phospholipids and triglycerides (14, 15). A previous study reported that IMF content is mainly determined by the number and size of intramuscular adipocytes (16). Furthermore, IMF is a complex quantitative trait, which is influenced by a variety of regulatory factors, such as gene regulation (17), sex (18), age, or body weight (19), as well as environmental conditions, cell signals, and diet (20). A previous study has shown that IMF plays a key role in many meat quality characteristics and quality (21). For example, IMF can improve meat quality by improving flavor, juiciness, and tenderness (22, 23). Therefore, IMF not only plays a very important role in animal husbandry production but also is closely related to a healthy and desirous human food supply. In recent years, the identification of candidate genes for IMF to improve meat quality has become an important research topic in livestock breeding. A large number of studies have investigated candidate genes affecting IMF content in many species, including cattle (24), pigs (25), sheep (26), and goats (27). Genes, such as PHKG1 (28), MYH3 (20), and PLIN1 (29), have been identified as candidates for regulating IMF content in pigs. However, candidate genes and regulatory mechanisms for IMF content in donkeys are not fully resolved.

With recent developments in proteomic technologies, it has become an increasingly important approach to identify candidate proteins related to meat quality in livestock. A previous study identified 127 proteins with differential abundance associated with IMF in the longissimus dorsi (LD) muscle of pigs between days 120 and 240 of growth (30). Hou et al. (31) identified proteins from the pig related to postmortem meat quality using a TMT (tandem-mass-tagged)-labeled quantitative proteomic. Similarly, proteomics was used to detect proteins associated with meat quality traits in other species including sheep (32), cattle (33), and chickens (34). Current proteomic studies on the donkey have mainly focused on the identification of proteins associated with donkey milk (35, 36). Few studies on proteins related to the regulation of donkey meat quality have been reported.

In this study, expression profiling of proteins was performed using LD muscle samples with divergent IMF content phenotypes (H and L IMF content groups). We used bioinformatic methods to identify the differentially abundant proteins (DAPs) between the H and L IMF groups. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DAPs was conducted. The aim of this study was to identify candidate proteins that influence IMF content in donkeys and to provide a basis for improving the quality of donkey meat.

Materials and methods

Ethics statement

All animal procedures described in this study were conducted according to the animal husbandry guidelines of the Shenyang Agricultural University. The studies on these animals were reviewed and approved by the Ethics Committee and Experimental Animal Committee of Shenyang Agriculture University (No. 202006032).

Sample preparation

All animals used in this study were derived from a population of 30 donkeys described in a previous study (37). These animals were raised under the same environmental conditions. At about 15 months of age, all donkeys were slaughtered in the same abattoir and LD muscle tissue samples were collected for IMF determination and protein extraction. Samples for protein extraction were immediately frozen in liquid nitrogen and then stored at −80°C until use. The Soxhlet extraction method (37) was used to determine the IMF content of the samples.

Protein extraction and digestion

Protein in the samples was isolated after disruption of the tissue in SDT [4% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, 1 mM DTT, pH 7.6] buffer, with the protein concentration quantified with the bicinchoninic acid (BCA) Protein Assay Kit (Bio-Rad, USA). Protein digestion was performed using trypsin according to the filter-aided sample
preparation (FASP) method previously described by Matthias Mann (38). Briefly, protein from each sample was incorporated into SDT [4% SDS, 100 mM dithiothreitol (DTT), 150 mM Tris-HCl pH 8.0] buffer. The detergent, DTT, and other low-molecular-weight components were removed using UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0). Iodoacetamide (IAA), 100 µl of 100 mM IAA in UA buffer, was added to block reduced cysteine residues and incubated in the dark for 30 min. The filter was then washed three times with 100 µl UA buffer and then twice with 100 µl 25 mM NH₄HCO₃ buffer. The eluted protein suspension was then digested overnight at 37°C with 4 µg trypsin (Promega) in 40 µl of 25 mM NH₄HCO₃ buffer and the resulting peptides were collected as filtrate. Digested peptides for each sample were desalted on C₁₈ cartridges, concentrated by vacuum centrifugation, and reconstituted in 40 µl of 0.1% (v/v) formic acid.

**Tandem-mass-tag labeling**

Peptides from the three high (H1, H2, and H3) and three low (L1, L2, and L3) IMF content donkey samples were labeled with 126, 127, 128, 129, 130, and 131 isotope tags, respectively, using TMT reagents according to the manufacturer's instructions (Thermo Scientific, Waltham, USA).

**Liquid chromatography–mass spectrometry analysis**

Liquid chromatography–mass spectrometry (LC–MS/MS) analysis was conducted on a Q Exactive mass spectrometer (Thermo Scientific, Waltham, USA). MS data were obtained using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1,800 m/z) for high-energy collisional dissociation (HCD) fragmentation. The automatic gain control (AGC) target was $3 \times 10^6$ and the maximum injection time was 10 ms. The dynamic exclusion duration was 40.0 s. Survey scans were obtained at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200, and isolation width was 2 m/z. The normalized collision energy was 30 eV and the underfill ratio was 0.1%.

**Identification and quantitation of proteins**

The MS raw data for each sample were searched against the donkey UniProt database using the MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 software for protein identification and quantitation. Proteins with fold change (FC) >1.2 or <0.833 and $p < 0.05$ were considered to be significantly DAPs.

**Gene ontology and KEGG pathway enrichment analysis of DAPs**

The gene ontology (GO) term annotation of selected DAPs was performed using Blast2GO software (39, 40). The DAPs were blasted against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) to retrieve their KEGG orthology identifications, which were subsequently mapped to the pathways in KEGG (41). GO and KEGG enrichment was analyzed using the Fisher's exact test. GO and KEGG terms with $p < 0.05$ were considered significantly enriched.

**Protein–protein interactions analysis**

For protein–protein interactions, we used STRING v11.5 (42) to predict and visualize networks according to default parameters. Protein interactions were illustrated using Cytoscape software (43).

**Results**

**Characterization of identified proteins**

We selected three LD muscle samples with high (H1, H2, and H3) and three with low (L1, L2, and L3) IMF content from a population of 30 individuals. Statistical analysis of the data showed that IMF content was significantly different between H and L samples (3.04 ± 0.12%, 6.39 ± 0.47%; $p < 0.01$). We further characterized the protein abundance profiles of the six samples using the TMT-labeled proteomic approach. The entire proteomic experimental flow for this study is shown in Figure 1. A total of 585,555 spectra were detected from the six LD muscle samples by LC–MS/MS analysis (Figure 2A). From this, we identified a total of 20,583 peptides, of which 15,279 were unique, corresponding to 2,540 distinct proteins (Figure 2A; Supplementary Table S1). A statistical analysis of the lengths of the identified peptide was conducted, which showed that the peptides were mainly between 5 and 15 amino acids with peptides of 7 and 9 amino acids in length being the highest (Figure 2B). Further analyses of the numbers of peptides identified in the proteins showed that about 50% of the proteins contained 1–3 identified peptides (Figure 2C). The molecular weights of the identified proteins indicated that most of these proteins have molecular weights between 10 and 70 kDa (Figure 2D).
Identification of DAPs between high and low IMF groups

To further explore candidate proteins associated with IMF deposition in donkeys, we analyzed the differentially abundant proteins in the high and low IMF content groups. A total of 30 DAPs were identified between the H and L IMF groups, with 13 upregulated and 17 downregulated in the H IMF group (Figure 3A). Summary information on these proteins is listed in Supplementary Table S2. The top upregulated DAP is MAP4K4, while the top downregulated DAP is Rho-associated protein kinase 2 (ROCK2) (Supplementary Table S2). We also examined the abundance patterns of these DAPs in the six samples, which showed clear abundance differences between the high and low IMF group samples (Figure 3B).

Gene ontology and KEGG enrichment analysis of DAPs

To explore the potential biological functions of the DAPs in IMF deposition, we performed GO and KEGG functional enrichment analyses of the DAPs. Here, 227, 27, and 24 GO terms were found to be significantly enriched in biological process, cellular component, and molecular function, respectively (Supplementary Table S3). The top 30 significantly enriched GO terms are shown in Figure 4A. This data suggests that the DAPs are primarily involved in bone morphogenetic protein (BMP) receptor binding, which is associated with lipid metabolism. The KEGG enrichment analysis suggested that the DAPs are significantly enriched in 19 pathways (Figure 4B). Interestingly, some of these KEGG participate in the lipogenic processes, for instance, the Wnt signaling and Hippo signaling pathways. Further, a DAP, ROCK2, is significantly enriched in the Wnt signaling pathway. These results suggest that the enrichment of DAPs in these pathways may be closely related to the lipogenic process.

Protein–protein interactions analysis for DAPs

Next, we examined protein–protein interactions of the 30 DAPs to better understand IMF deposition. A protein–protein interaction analysis of the DAPs was conducted based on the STRING database, which contains details functional relationships between proteins, thus allowing predictions on the functional impact of changing protein abundance (44). The results of this analysis showed that 16 DAPs are involved in a protein–protein interaction network (Figure 5). For example, a DAP, ROCK2, interacts with UBR4, TJP1, MBP, and PTEN. In addition, the ADGRV1 and USH2A proteins interact with each other.

Discussion

In recent years, consumers have become increasingly interested in donkey meat, with China becoming the world’s largest consumer of this meat (45). IMF plays a vital role in the quality of livestock meat, therefore, the identification of candidate proteins affecting IMF is essential for improving meat quality. Previous studies have applied proteomics to identify DAPs of IMF in other species including pigs (30) and goats (46). However, there is a paucity of research on candidate proteins for quality traits in donkey meat. In this study, we identified 30 proteins that were differentially abundant between LD muscle samples with high and low IMF content based on proteomic techniques. Our results suggest that the identified DAPs are candidates for influencing IMF content in donkey meat.

Further, of these DAPs, many are associated with the adipogenic processes. For example, ROCK2, a downregulated protein, inhibits adipogenesis based on knockdown experiments (47). Protein interaction analysis revealed that ROCK2 potentially binds UBR4, TJP1, MBP, and PTEN proteins. These results suggest that the ROCK2 protein might act as a suppressor of IMF deposition in the donkey by binding these proteins.
Ahbara et al. (48) revealed the SPAG8 gene is a candidate for growth traits and adipogenesis in sheep. A previous study identified the RPL27A gene as a candidate gene for bovine marbling, with a single nucleotide polymorphism (SNP) in its promoter used as a molecular marker for bovine marbling (49). Tu et al. (50) found that a high-fat diet increases the expression level of TPM1. Another interesting finding is that PRMT3 acts as a co-transcription factor by translocating into the nucleus and binding to LXRα to regulate downstream gene expression, thereby promoting lipogenesis (51). These proteins (i.e., SPAG8, RPL27A, TPM1, MBP, and PRMT3) were identified as DAPs between the H and L IMF groups in this study, suggesting that they are important regulators of IMF. However, the mechanism of action of these DAPs in IMF deposition in donkeys needs to be further demonstrated.

Our study identified a number of GO terms related to the lipogenic process based on enrichment analysis of the DAPs, including tRNA methylation and BMP receptor binding. Zhao et al. (52) showed that RNA methylation has a crucial role and is required for adipose differentiation. In addition, it has been shown that tRNA methylation levels regulate the translation initiation of genes and thus affect protein synthesis (53). These data suggest that some DAPs may regulate IMF deposition by affecting tRNA methylation. BMP signaling is essential for the differentiation of mesenchymal stem cells (MSCs) into the adipocyte lineage (54). Jung et al. (55) found that plasma BMP2 levels were positively correlated with IMF content in steers, suggesting that BMP signaling contributes to meat quality. In addition, a GO term, positive regulation of c-Jun N-terminal kinase (JNK) cascade, was significantly enriched in this study. A previous study demonstrated that JNK signaling inhibits adipose differentiation by suppressing peroxisome proliferator-activated receptor-gamma (PPARγ) and fatty acid binding protein 4 (AP2) expression (56). Taken together, these DAPs may regulate the deposition of IMF through an association with adipogenesis.

In addition, DAPs were enriched in many key signaling pathways (e.g., Wnt and Hippo signaling pathways) involved in the adipogenic process. The Wnt signaling pathway is a highly conserved critical factor in animals that negatively regulates adipose differentiation (57). Mechanistically, Wnt signaling
FIGURE 3
Analysis of differentially abundant proteins (DAPs) between high and low intramuscular fat (IMF) groups. (A) Volcano plot showing DAPs between high and low IMF groups. Red, gray, and blue dots represent upregulated, unchanged, and downregulated proteins, respectively. (B) Heatmap showing the abundance patterns of the DAPs between the three high and three low IMF samples.
Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis of differentially abundant proteins (DAPs). (A) Top 30 significantly enriched GO terms. The X-axis represents the $-\log_{10}(p\text{ value})$ value of the GO terms and the Y-axis represents the GO term name. (B) Significantly enriched pathways of DAPs. The X-axis represents the rich factor and the Y-axis represents the pathway. The size and color of the bubbles represent the number of proteins enriched in the pathway and enrichment significance, respectively.
Protein–protein interactions analysis for differentially abundant proteins (DAPs). The red nodes represent DAPs, while the green nodes represent other proteins that interact with DAPs. The edge represents interactions between proteins.

Inhibits adipogenesis by suppressing the expression of PPARγ and CCAAT/enhancer-binding protein alpha (C/EBP)α (58). Activation of Wnt signaling, by transgenic overexpression of Wnt10, in mice leads to significantly reduced adipose tissue weight (59). A downregulated protein identified in our study, ROCK2, is significantly enriched in the Wnt signaling pathway, suggesting that it inhibits IMF deposition by mediating the Wnt signaling pathway. The Hippo signaling pathway was also enriched, and it has been shown to be involved in adipocyte proliferation and differentiation in animals (60, 61). In Hippo signaling pathway, serine/threonine kinase 24 (Ste20) family kinases mammalian STE20-like kinase (MST1/2) activate large tumor suppressor (LATS) kinases through phosphorylation, which in turn allows phosphorylation of yes-associated protein (YAP) protein and facilitates the binding of YAP to 14-3-3 proteins (62). Park et al. (63) found that MST1/2 promotes the differentiation of 3T3-L1 cells through the activation of PPARγ. Deng et al. (61) demonstrated that YAP1 inhibits the differentiation of ovine adipocytes by affecting PPARG and RXR alpha levels. These results suggest that the Hippo signaling pathway is implicated in the deposition of IMF in the donkey. However, how DAPs involved in Hippo signaling regulate the deposition of IMF in donkeys still needs to be further explored.

Conclusion

In summary, we identified 30 candidate proteins that might affect IMF content in the LD muscle of donkeys. We provide evidence that some of the DAPs affect IMF deposition as they are involved in adipogenic functions or signaling pathways in other animals. Our data provide evidence for the role of these proteins in IMF content in the donkey and provide new insights into the molecular mechanisms of the regulation of IMF deposition.
Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee and Experimental Animal Committee of Shenyang Agriculture University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BL and SZha conceived and designed this study. YH, YQ, and XT performed the experiments. ZY, JC, SZho, and RZ analyzed the data. XT and BL drafted the manuscript. DI and BL revised the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.951168/full#supplementary-material

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