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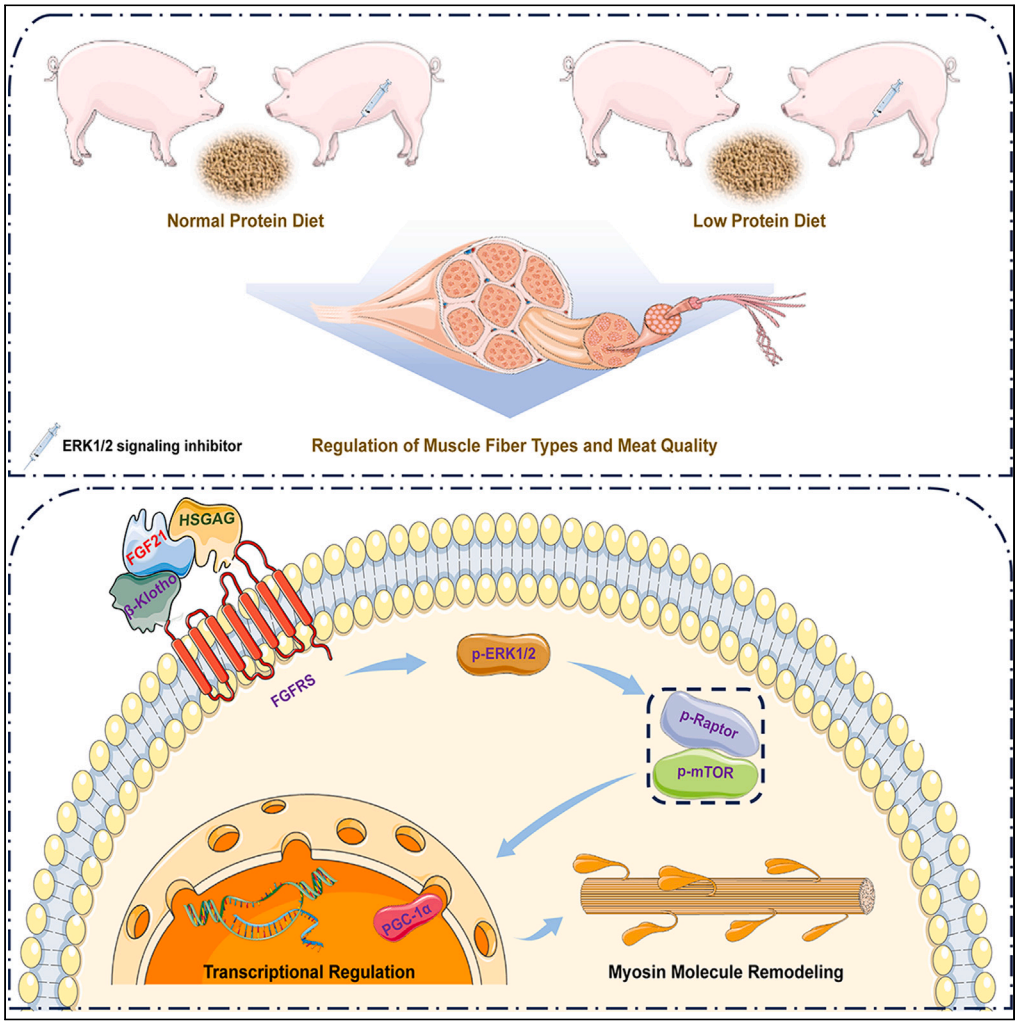

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Highlights

Dietary protein restriction affects skeletal muscle metabolic characteristics in pigs

Dietary protein restriction promotes slow myofiber-type transformation

Dietary protein restriction induces FGF21 expression in skeletal muscle

Skeletal muscle fiber remodeling is mediated by FGF21-ERK1/2-mTORC1 signaling

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Dietary protein restriction regulates skeletal muscle fiber metabolic characteristics associated with the FGF21-ERK1/2 pathway

Shuo Li,[1] Haopeng Zhong,[1] Zirui Wang,[1] Jun Chen,[1] Zhouyin Huang,[1] Tiande Zou,[1,*] and Jinming You[1,2,*]

SUMMARY

Under conditions of dietary amino acid balance, decreasing the dietary crude protein (CP) level in pigs has a beneficial effect on meat quality. To further elucidate the mechanism, we explored the alteration of muscle fiber characteristics and key regulators related to myogenesis in the skeletal muscle of pigs fed a protein restricted diet. Compared to pigs fed a normal protein diet, dietary protein restriction significantly increased the slow-twitch muscle fiber proportion in skeletal muscle, succinic dehydrogenase (SDH) activity, the concentrations of ascorbate, biotin, palmitoleic acid, and the ratio of s-adenosylhomocysteine (SAM) to s-adenosylhomocysteine (SAH), but the fast-twitch muscle fiber proportion, lactate dehydrogenase (LDH) activity, the concentrations of ATP, glucose-6-phosphate, SAM, and SAH in skeletal muscle, and the ratio of serum triiodothyronine (T3) to tetraiodothyronine (T4) were decreased. In conclusion, we demonstrated that dietary protein restriction induced skeletal muscle fiber remodeling association the regulation of FGF21-ERK1/2-mTORC1 signaling in weaned piglets.

INTRODUCTION

Skeletal muscle development may contribute to affecting pork quality evaluation. The characteristics of skeletal muscle fibers are critical indexes for evaluating the overall condition of skeletal muscle.[1,2] Based on oxidative metabolic capacity and contraction rate, skeletal muscle fibers can be divided into slow-oxidative (type I), fast-oxidative (type IIa), fast-intermediate (type IIx) and fast-glycolytic (type IIb).[2] Skeletal muscle fiber characteristics are mainly determined by a critical balance between the number and type of muscle fibers.[2,3] However, this balance is broken in conditions of chronic stress, as substantial research has demonstrated that long-term ingestion of certain exogenous substances can induce the conversion of skeletal muscle fiber characteristics.[3–5] Notably, the quality of muscle rich in oxidative muscle fibers is much higher than that of muscle with a high proportion of glycolytic muscle fibers.[2] Moreover, maintaining excellent functional performance and normal development of skeletal muscle is essential for signal transduction, energy metabolism and animal meat quality.[6] In addition, understanding the molecular mechanisms underlying muscle fiber conversion is a key step in elucidating the regulatory mechanisms of pork quality.

Recent research has indicated that fibroblast growth factor (FGF), complexes composed of its receptors are involved in controlling biological function occurrence such as cellular growth, proliferation, and metabolic programs.[7] Of the 22 subtypes of FGFs, FGF21 is a stress-related protein molecule that regulates lipid metabolism, glycogen homeostasis, and muscle development.[8–10] Moreover, as a myogenic regulator closely related to muscle metabolism, FGF21 secretion is an adaptive regulatory measure that regulates metabolic homeostasis in muscle, especially in the context of stresses, such as muscle mitochondrial dysfunction and endoplasmic reticulum stress.[11,12] FGF21 plays a role in cardiomyocyte function, as manifested by increased cardiac fibrosis deposition and mitochondrial morphology damage in an FGF21-KO mouse model of diabetic cardiomyopathy. FGF21 deficiency leads to elevated accumulation of collagen fibers and mitochondrial dilatation, membrane distortion and cristae deletion, enhancing susceptibility to cardiac fibrosis and mitochondrial function damage.[12] Furthermore, FGF21/ERK signaling is a key perspective for alleviating skeletal muscle injury.[13] There is evidence that ERK1/2-mediated activation of myosin heavy chain (MyHC) I/β expression is essential for skeletal muscle fiber type remodeling.[14] On the other hand, many studies have demonstrated that FGF21 also mediates the skeletal muscle mitochondrial respiratory chain to increase oxygen consumption and energy metabolism efficiency by stimulating mTOR phosphorylation and the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α).[15] In addition, mechanistic target of rapamycin complex 1 (mTORC1) activation in response to changes in amino acid intake is accompanied by increased secretion of FGF21.[16] Although FGF21-mediated improvements in muscle development have been extensively researched, the molecular regulatory ability to modulate both muscle fiber type transformation and differentiation is still poorly understood.

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It is of great importance to achieve a deeper understanding of the biological processes affected by modifying dietary proteins that influence meat quality control. In this sense, extensive numbers of studies have demonstrated that different dietary protein levels could affect muscle protein deposition and muscle properties.[17,18] High protein intake contributes to mitigating adverse effects on muscle caused by a high-fat diet and improving muscle mass and functional performance.[19] However, a high protein diet does not increase basal muscle protein synthesis rates, suggesting that high protein supplementation may not be fully converted to muscle protein.[20] Conversely, a low protein diet is a nutritional strategy to reduce the level of CP in the diet without affecting the growth performance and product quality of animals, and at the same time to meet the needs of animals by adding the appropriate type and number of amino acids,[21] such as four limiting amino acids (L-lysine, L-methionine, L-threonine, and L-tryptophan) need to be supplemented in a low-protein diet for pigs.[22] Decreased protein intake promotes the activation of amino acid transporters and regulates the free amino acid profile, altering the expression of various transcription factors that control muscle development in pigs, especially the mTOR-related pathway and its downstream signaling.[17,23] In addition, patterns of dietary protein restriction with balanced amino acids may also improve meat quality by regulating intramuscular fat content.[22] Although dietary protein restriction has been demonstrated to be an appropriate means to regulate muscle metabolism and meat quality, its effect on skeletal muscle fiber type transformation, a critical metabolism-related problem, remains elusive.

Previous studies have indicated that FGF21 expression was elevated in the liver, adipose tissue, and muscle of mice, and this increase was associated with dietary protein restriction.[24,25] Therefore, it is hypothesized that improvements in meat quality and changes in muscle development regulation-related genes in pigs are mediated by FGF21, whose expression is induced by dietary protein restriction. To verify this hypothesis, in the present study, we aimed to investigate the effects of a protein restricted diet on skeletal muscle fiber characteristics and the expression of key molecules associated with the FGF21-ERK1/2-mTORC1 pathway in weaned piglets.

RESULTS

Dietary protein restriction decreased skeletal muscle weight and changed the proportions of different types of muscle fibers

We adopted a strategy of reducing dietary protein levels to study the effects of dietary protein levels and ERK1/2 inhibitors on the skeletal muscle characteristics of weaned piglets. Regardless of ERK1/2 inhibitor administration, dietary protein restriction significantly reduced skeletal muscle weight ($p < 0.05$, Figure 1A). Next, we performed ATPase staining to evaluate the different fiber type compositions of skeletal muscle in weaned piglets. Our results showed that skeletal muscle was made up primarily of fast-twitch muscle fibers such as MyHC IIa and IIb, and slow-twitch muscle (MyHC I) fibers made up approximately 40% (Figures 1B and 1C). Notably, regardless of ERK1/2 inhibitor administration, pigs fed a low protein diet exhibited a significant increase in oxidative (MyHC I and IIa) and a concomitant reduction in fast muscle glycolytic (MyHC IIb) fibers in skeletal muscle compared with a normal protein diet ($p < 0.05$, Figures 1B and 1C). Furthermore, ERK1/2 inhibitor administration significantly reduced the proportion of MyHC IIa fibers while upregulating the proportion of MyHC IIb fibers ($p < 0.05$, Figures 1B and 1C). In addition, there was no difference in pig muscle fiber density under either dietary protein restriction or ERK1/2 inhibitor treatment (Figure 1D). Taken together, these findings suggest that different muscle fiber type proportions were significantly changed by dietary protein restriction and ERK1/2 inhibitor treatment. These results also indicate that muscle weight regulation may not be associated with ERK1/2 inhibitors.

Serum T3 and T4 concentrations and muscle metabolic enzyme activities were regulated by dietary protein restriction

Given that weaned piglets treated with dietary protein restriction and ERK1/2 inhibitor showed altered skeletal muscle fiber characteristics, we next investigated skeletal muscle metabolic enzyme activity and related serum parameters. Decreasing dietary protein levels reduced the concentration of T3 ($p < 0.05$) and the ratio of T3 to T4 ($p < 0.05$) in serum in weaned piglets (Figures 2A and 2C). In contrast, ERK1/2 inhibitor administration increased the concentration of T3 ($p < 0.05$) and the ratio of T3 to T4 ($p < 0.05$) in the serum of weaned piglets (Figures 2A and 2C). However, there was no difference in serum T4 content under either dietary protein restriction or ERK1/2 inhibitor treatment (Figure 2B). In addition, SDH activity was significantly increased with dietary protein restriction ($p < 0.05$), and ERK1/2 inhibitor treatment significantly reduced SDH activity ($p < 0.05$, Figure 2D). Notably, CK activity was not affected by different protein levels but was significantly reduced in skeletal muscle by ERK1/2 inhibitor administration ($p < 0.05$, Figure 2E). LDH activity was significantly decreased with dietary protein restriction ($p < 0.05$), and ERK1/2 inhibitor treatment significantly increased LDH activity ($p < 0.05$, Figure 2F). Regardless of ERK1/2 inhibitor treatment, pigs offered lower dietary protein levels had significantly reduced LA content in skeletal muscle ($p < 0.05$, Figure 2G).

Dietary protein restriction promoted skeletal muscle fiber type remodeling

To verify the changes seen based on the conversion effects of dietary protein restriction and ERK1/2 inhibitor treatment on the skeletal muscle fiber types of weaned piglets, we then analyzed the expression of MyHC and genes associated with muscle development at the molecular level by western blot and qPCR. A low protein diet increased the mRNA expression of *Pax3* ($p < 0.05$) and an interaction tendency between dietary protein level and ERK1/2 inhibitor administration on *Pax3* mRNA expression was observed ($p = 0.065$, Figure 3A). Reducing dietary protein levels increased the mRNA expression of *Pax7* ($p < 0.05$), whereas ERK1/2 inhibitor administration tended to decrease *Pax7* mRNA expression ($p = 0.081$, Figure 3A). Regardless of ERK1/2 inhibitor treatment, decreasing dietary protein levels increased the mRNA expression of *Desmin* and *Myoglobin* and reduced the mRNA expression of *MyoD* ($p < 0.05$, Figure 3A). Notably, the protein expression of Myoglobin

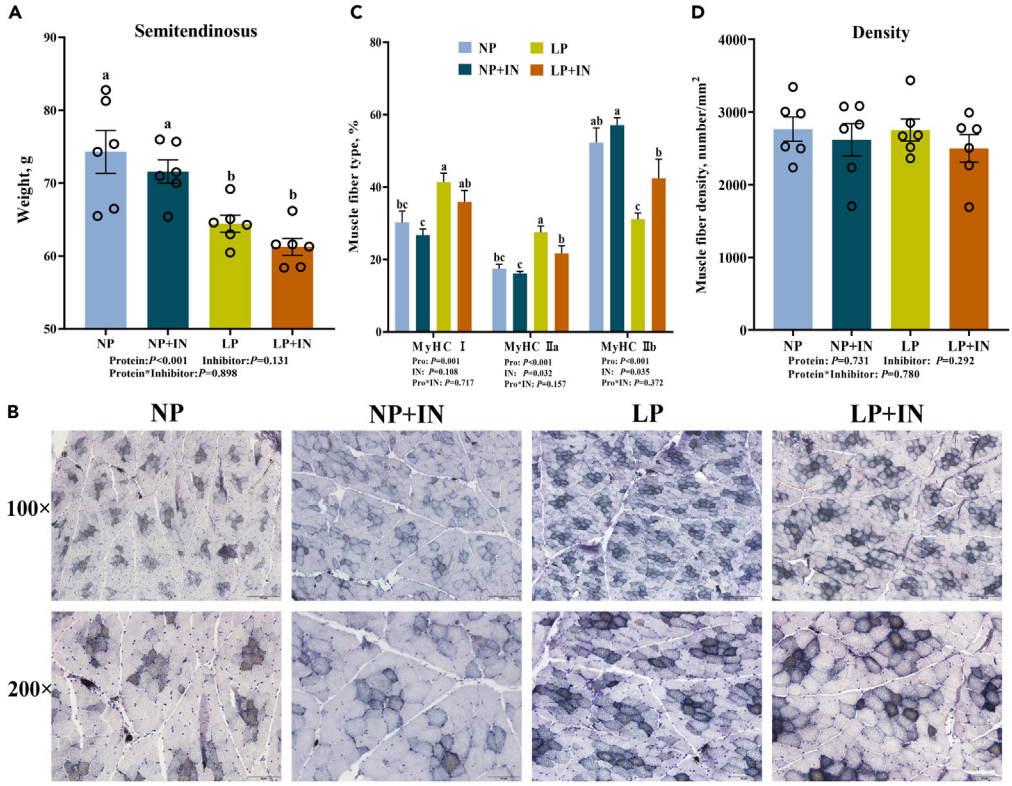

Figure 1. Effects of dietary protein restriction and ERK1/2 inhibition on skeletal muscle characteristics

(A) Changes in skeletal muscle weight induced by dietary protein restriction.

(B) Representative images of ATPase staining in skeletal muscle. The darkest stain represents myosin heavy chain (MyHC) I, the lightest stain represents MyHC IIb, and the middle is MyHC IIa. Scale bar = 100 μm (100×), Scale bar = 50 μm (200×).

(C) The proportions of different muscle fiber types in skeletal muscle.

(D) The conditions of skeletal muscle fiber density. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 6. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

tended to increase, regardless of ERK1/2 inhibitor treatment ($p = 0.067$, Figures 3C and 3F). However, ERK1/2 inhibitor administration decreased the mRNA expression of *Myf5* regardless of the dietary protein levels ($p < 0.05$, Figure 3A). *Myogenin* mRNA expression was significantly increased with dietary protein restriction ($p < 0.05$), and ERK1/2 inhibitor treatment significantly reduced *Myogenin* mRNA expression ($p < 0.05$, Figure 3A).

In addition, decreased dietary protein levels increased the mRNA expression of *MYH2* and *MYH7* and reduced the expression of *MYH4*, whereas ERK1/2 inhibitor treatment decreased the expression of *MYH2* and *MYH7* and increased the expression of *MYH4* ($p < 0.05$, Figure 3B). Neither dietary protein levels nor ERK1/2 inhibitor treatment impacted *MYH1* mRNA expression ($p > 0.05$, Figure 3B). Similarly, we found that dietary protein restriction significantly reduced the expression of fast-MyHC and increased the expression of slow-MyHC ($p < 0.05$, Figures 3C–3E). Notably, ERK1/2 inhibitor administration increased the expression of fast-MyHC and reduced the expression of slow-MyHC ($p < 0.05$, Figures 3C–3E). Taken together, the results imply that dietary protein restriction may improve muscle development and promote slow-muscle fiber formation through ERK1/2 signaling.

Dietary protein restriction significantly induced FGF21 expression

Since dietary protein restriction and ERK1/2 inhibitor administration remodeled the muscle fiber composition, we used dietary protein restriction to induce muscle FGF21 production and further explored the role of FGF21 in muscle fiber type transformation in weaned piglets. The expression of FGF21 was determined using qPCR and western blotting analysis in skeletal muscle in weaned piglets. Notably, we observed that dietary protein restriction treatment enhanced the mRNA expression of *FGF21* in skeletal muscle ($p < 0.05$), regardless of ERK1/2 inhibitor administration (Figure 4A). Moreover, the FGF21 protein levels remained high in piglets that underwent dietary protein restriction ($p < 0.05$, Figures 4B and 4C). However, ERK1/2 inhibitors are not involved in the regulation of FGF21 secretion. Collectively, these results indicate that dietary protein restriction significantly upregulated FGF21 expression in skeletal muscle in weaned piglets.

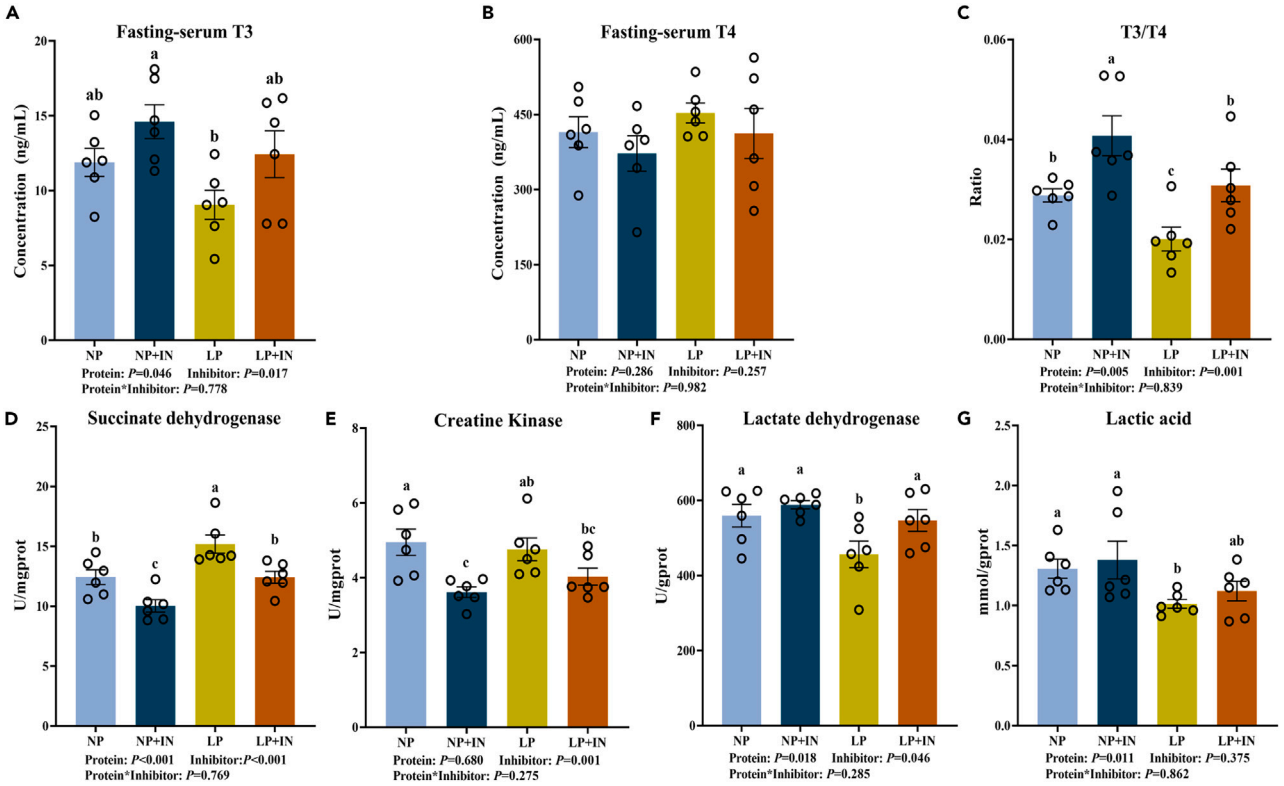

Figure 2. Effects of dietary protein restriction and ERK1/2 inhibitor administration on serum T3 and T4 content and muscle metabolic enzyme activities in weaned piglets

(A) The concentration of T3 in serum.

(B) The concentration of T4 in serum.

(C) The ratio of T3 to T4 levels.

(D) The changes of SDH activity in skeletal muscle in weaned piglets.

(E) The change of CK activity in skeletal muscle in weaned piglets.

(F) The change of LDH activity in skeletal muscle in weaned piglets.

(G) The change of lactic acid (LA) content in skeletal muscle in weaned piglets. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 6. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

Dietary protein restriction activated ERK1/2-mTORC1 signaling and is associated with the upregulation of FGF21 expression

To investigate whether dietary protein restriction was the major reason for muscle fiber type transformation, ERK1/2 signaling was analyzed by western blotting in weaned piglets. Notably, in line with FGF21 expression, we found that the phosphorylation of ERK1/2 and the ratio of phosphorylated ERK1/2 to ERK1/2 in skeletal muscle were elevated in dietary protein restriction conditions in weaned piglets compared to normal protein treatment ($p < 0.05$, Figures 5A and 5B), whereas ERK1/2 inhibitor administration significantly blocked the phosphorylation of ERK1/2 and decreased the ratio of phosphorylated ERK1/2 to ERK1/2, regardless of dietary protein levels ($p < 0.05$, Figures 5A and 5B). In line with the increased activation of ERK1/2 signaling, dietary protein restriction treatment tended to increase the phosphorylation of mTOR ($p = 0.08$, Figures 5A and 5C) and significantly increased the ratio of phosphorylated mTOR to mTOR, whereas ERK1/2 inhibitor administration significantly decreased the phosphorylation of mTOR and the ratio of phosphorylated mTOR to mTOR ($p < 0.05$, Figures 5A and 5C). In addition, we found that a lower protein diet significantly increased the ratio of phosphorylated Raptor to Raptor, and ERK1/2 inhibitor administration significantly decreased the ratio of phosphorylated Raptor to Raptor ($p < 0.05$, Figures 5A and 5D). Regardless of dietary protein levels, ERK1/2 inhibitor administration significantly decreased the phosphorylation of S6 and the ratio of phosphorylated S6 to S6 ($p < 0.05$, Figures 5A and 5E). Furthermore, an increase in PGC-1α protein expression was observed in piglets fed a lower protein diet treatment, regardless of ERK1/2 inhibitor administration ($p < 0.05$, Figures 5A and 5F). These findings suggest that dietary protein restriction may hyperactivate the mTORC1/PGC-1α signaling cascade via increased phosphorylation of ERK1/2 to regulate muscle fiber transformation.

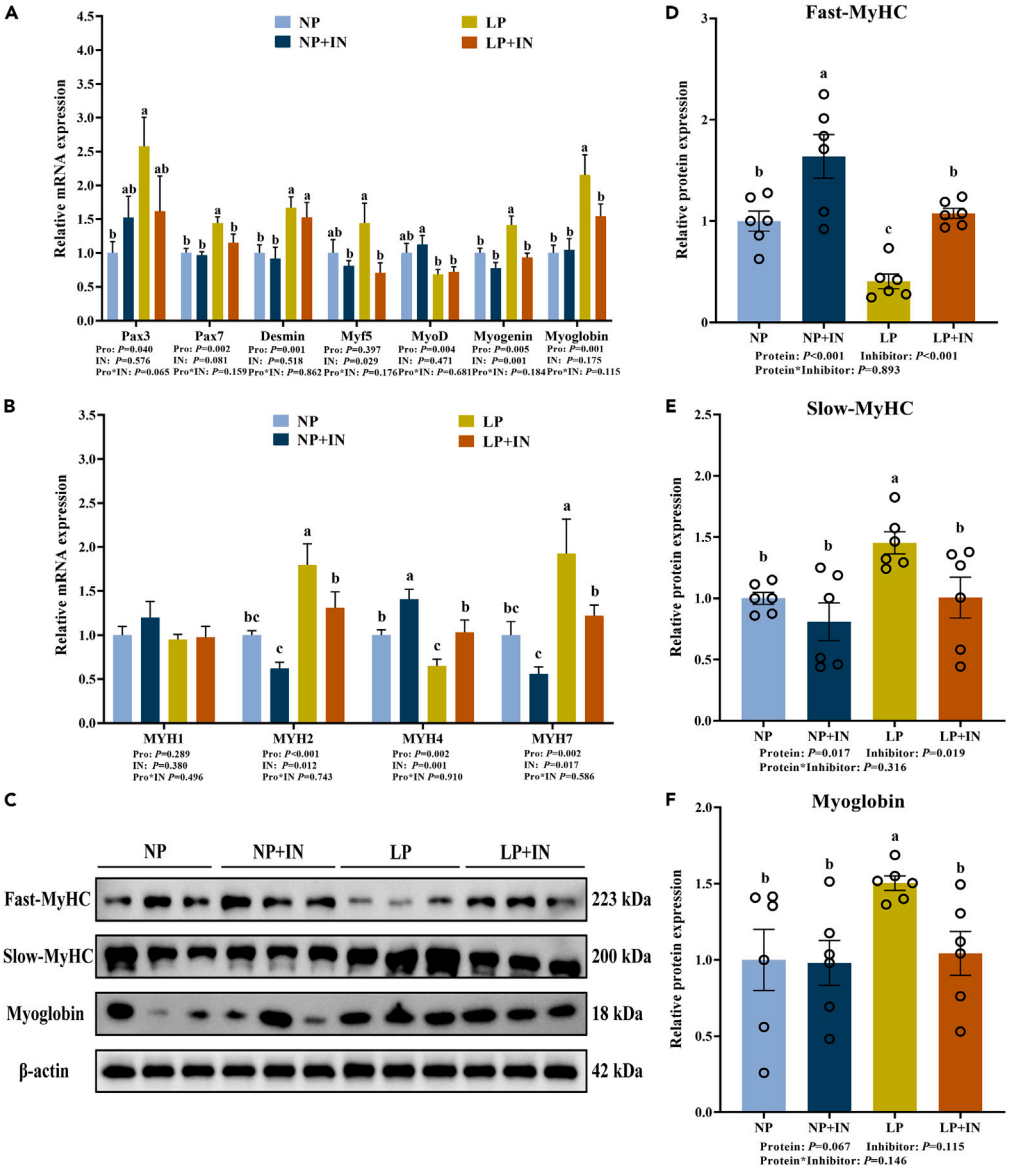

Figure 3. Effects of dietary protein restriction and ERK1/2 inhibitor treatment on skeletal muscle fiber transformation and muscle growth-related gene expression

(A) Relative mRNA expression of factors associated with muscle development.

(B) Relative mRNA expression of *MyHC* in skeletal muscle.

(C–F) Western blotting images and relative quantification to identify the expression of fast-MyHC, slow-MyHC, and myoglobin in skeletal muscle. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 6. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

Liquid chromatography-mass spectrum (LC-MS) metabolomic analysis of skeletal muscle in weaned piglets

To better understand the mechanisms of muscle fiber type remodeling, metabolomic analysis was conducted in skeletal muscle from weaned piglets under both dietary protein restriction and ERK1/2 inhibitor treatment. Representative total ion chromatograms (TIC) of skeletal muscle samples with similar trends demonstrated that the samples were reproducible and that the results were reliable (Figure S1A). Moreover, to obtain reliable and high-quality metabolomics data, quality control (QC) was conducted, and principal component analysis (PCA) showed a dense distribution of QC samples, indicating reliable data quality (Figure S1B). In addition, the proportion of the relative standard deviation (RSD) of the potential characteristic peak was less than 30% in the QC samples and reached 82.3% (Figure S1C). These results demonstrate that the metabolomic data check passed successfully.

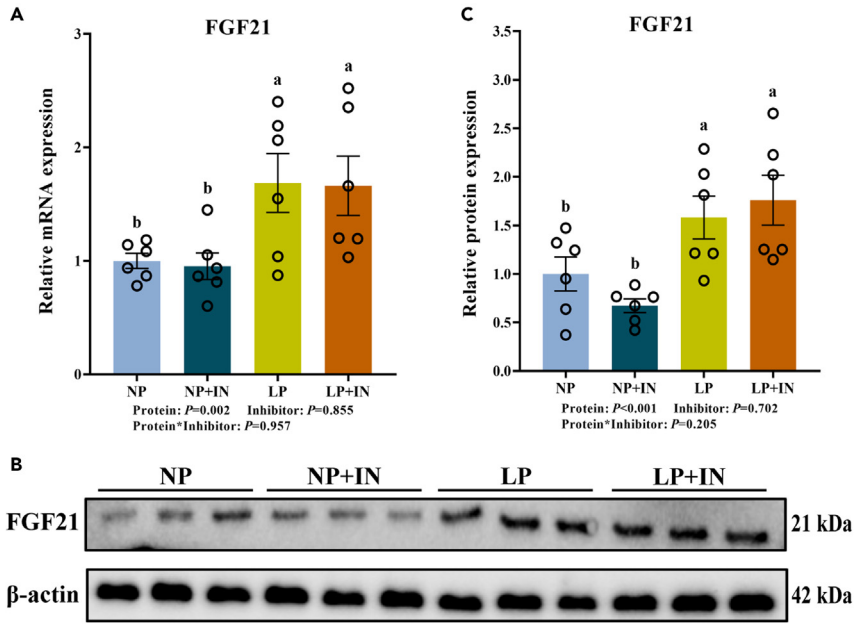

Figure 4. FGF21 levels increased in skeletal muscle in weaned piglets under dietary protein restriction

(A) Relative mRNA expression of *FGF21* in skeletal muscle.

(B and C) Western blotting images and relative quantification to identify FGF21 expression in skeletal muscle. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 6. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

Multivariate statistical analysis was used to classify the collected multidimensional data. The PCA score maps for all treatment groups were appropriately aggregated within limits and separated between groups in the original state of the metabolome data (Figure 6A), and R2X > 0.5 demonstrates that the model has a strong explanatory degree. Next, the OPLS-DA model was established to maximize the difference between groups and clearly showed differences between the groups (Figure 6B). Simultaneously, to evaluate the effectiveness of the model and prevent overfitting, permutation tests were conducted on the four groups. The results of permutation tests showed that R2 (green line) was higher than Q2 (blue line), and the intercept between the regression line and Y axis was less than 0, indicating that there was no overfitting phenomenon in these models (Figure 6C). Collectively, these findings suggested that multivariate statistical analysis can reliably reflect the state of the original data between groups.

Identification of differential metabolites (DM)

To better identify the differences between different processing methods, we performed MS to identify DM based on a variable importance in projection (VIP) score >1 obtained by OPLS-DA and $p < 0.05$. A total of 372 metabolites were identified by metabolomic analysis (Table S1). Consequently, 55, 39, 35, 59, and 46 DM were identified in the NP-IN vs. NP, LP-IN vs. NP-IN, LP-IN vs. LP, LP vs. NP, and NP-IN vs. NP vs. LP-IN vs. LP groups, respectively (Table S1). Next, hierarchical cluster analysis was used to determine the different patterns of metabolites under different dietary protein levels and ERK1/2 inhibitor treatments. Moreover, we performed hierarchical cluster analysis on the four groups by pairwise comparison. These data suggest that the clustering effect of DM was better within the same group, and the difference between groups was obvious (Figure 7). Although there was one abnormal sample in the LP vs. LP-IN group, it does not affect the overall clustering effect (Figure 7C). Taken together, these observations suggest that dietary protein levels and ERK1/2 inhibitor could affect the type and number of DM in skeletal muscle.

The effects of dietary protein restriction and ERK1/2 inhibition on key DM associated with muscle fiber type

Skeletal muscle fiber type conversion was activated under both a low protein diet and ERK1/2 inhibitor treatment. Then, DM was analyzed, and the relationship between the production of DM and muscle fiber type conversion was explored. By classifying and screening all the different metabolites in the NP-IN vs. NP vs. LP-IN vs. LP groups, the effects of different dietary protein levels and ERK1/2 inhibitor treatment on muscle fiber type and pork quality were mainly determined from the aspects of muscle antioxidant capacity, intramuscular fat content, postmortem muscle glycolysis, muscle flavor substances and DNA methylation. Notably, the representative DM include ascorbate, ATP, biotin, glucose-6-phosphate, oleic acid, palmitoleic acid, SAM, and SAH (Figure 8). Compared with the NP groups, increased levels of ascorbate, biotin, palmitoleic acid and the ratio of SAM to SAH were observed in the LP groups, but levels of ATP, glucose-6-phosphate, SAM, and

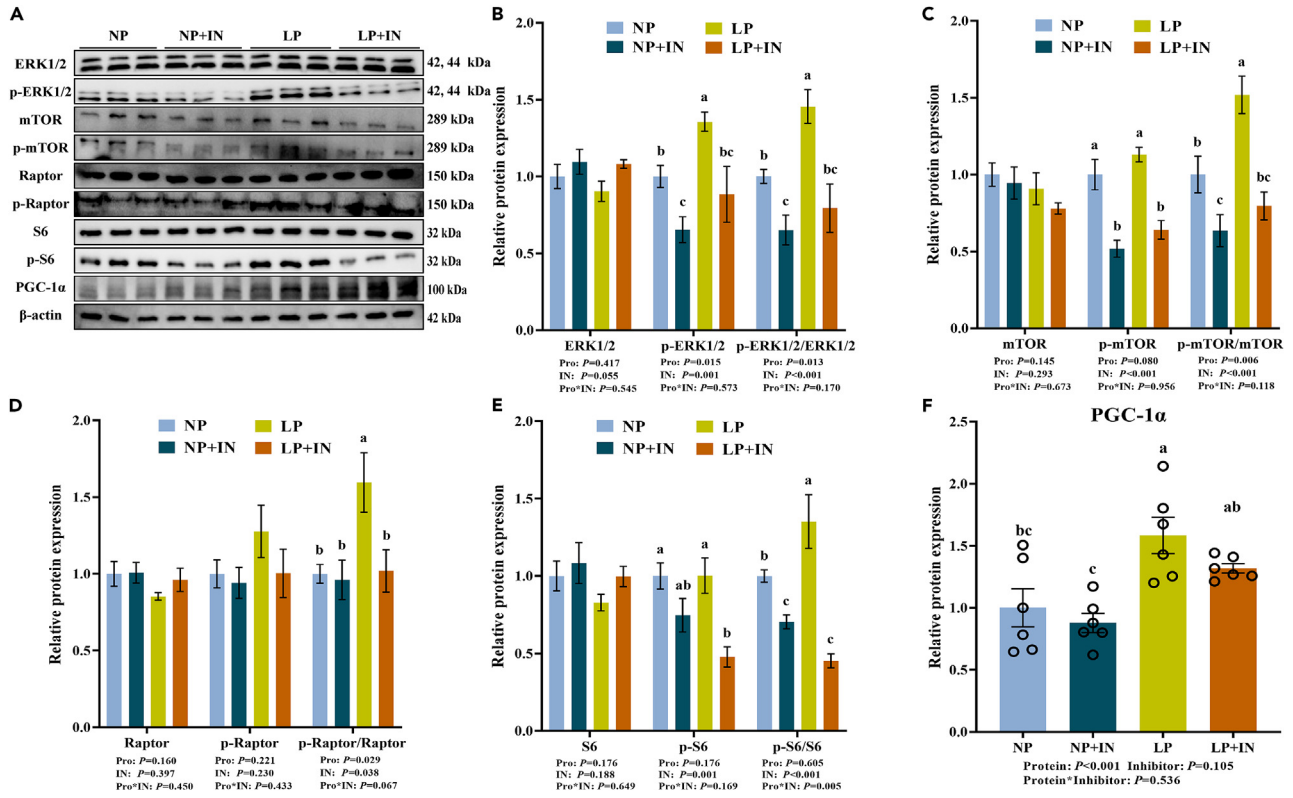

Figure 5. Dietary protein restriction activated ERK1/2-mTORC1 signaling in skeletal muscle in weaned piglets

(A) Western blotting images of total ERK1/2, mTOR, Raptor and S6, phosphorylated protein levels of ERK1/2, mTOR, Raptor and S6, and PGC-1α protein expression.

(B–F) Quantification of the above proteins. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 6. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

SAH were decreased ($p < 0.05$, Figure 8). In addition, ERK1/2 inhibitor administration decreased ascorbate and oleic acid content and increased ATP content with a normal protein diet ($p < 0.05$, Figure 8). Furthermore, ERK1/2 inhibitor administration increased ATP, glucose-6-phosphate, SAM, and SAH levels and decreased palmitoleic acid levels with a lower protein diet ($p < 0.05$, Figure 8). These data demonstrated that the changes in skeletal muscle DM of piglets treated with different dietary protein levels and inhibitors was related to the conversion of muscle fiber types.

Analysis of KEGG pathways

To explore metabolic pathways potentially affected by dietary protein restriction and ERK1/2 inhibitor treatment, all DM in the NP-IN vs. NP vs. LP-IN vs. LP groups were annotated by KEGG pathway analysis. We found that 18 major KEGG pathways were enriched, especially the hypoxia-inducible factor-1 (HIF-1) signaling pathway, calcium signaling pathway, biotin metabolism, biosynthesis of unsaturated fatty acids, oxidative phosphorylation and thyroid hormone synthesis. These data indicate that the metabolism of DM under the conditions of a low protein diet and ERK1/2 inhibitor administration was mainly mediated by these pathways (Figure 9).

DISCUSSION

Dietary protein levels play an important role in the regulation of muscle growth and development in livestock and poultry, and protein restriction has a significant effect on muscle fiber characteristics (muscle fiber types and metabolic patterns), which contribute to the improvement of meat quality.[17,18] In addition, a dietary protein restriction diet with balanced essential and nonessential amino acid can improve growth performance and muscle development.[23,26] This may explain the widespread application of dietary protein restriction strategies.[22] However, the mechanism of regulating muscle metabolism by reducing dietary protein levels still needs to be further explored. In the present study, we demonstrated that dietary protein restriction regulated skeletal muscle development and stimulated the conversion of muscle fibers from fast-twitch muscle to slow-twitch muscle, which may be associated with the activation of FGF21-ERK1/2-mTORC1 signaling.

The phenotypic differences in skeletal muscle fiber types are mainly between fast and slow muscle fibers or between glycolytic and oxidative muscle fibers. This difference is due to the differential translation and expression of genes that dominate different muscle fiber types.[2,3]

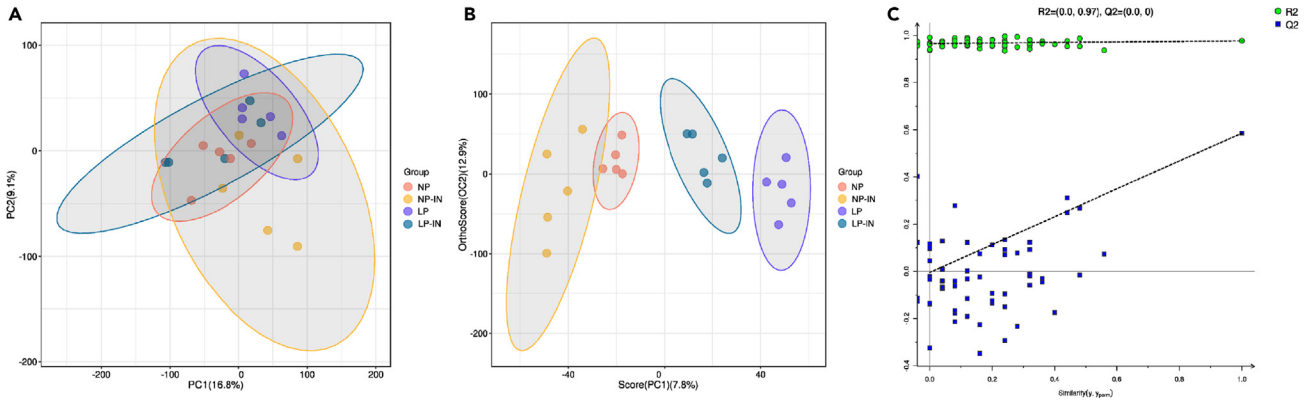

Figure 6. PCA and orthogonal projections to latent structures discriminant analysis (OPLS-DA) analysis from LC-MS profiles of skeletal muscle from weaned piglets under dietary protein restriction and ERK1/2 inhibitor treatment

(A) PCA score plot. R2X = 0.517.

(B) OPLS-DA score plot.

(C) OPLS-DA permutation test. R2Y = 0.977 and Q2 = 0.586. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 5.

Interestingly, this rearrangement is susceptible to external stimuli or internal physiological changes.[3] For example, plant-derived nutrients (such as quercetin and L-theanine) significantly alter the proportions of fast and slow muscle, increase the proportions of oxidized muscle fibers and decrease the proportions of glycolytic muscle fibers in skeletal muscle in mice.[4,5] Therefore, it is feasible to regulate muscle fiber type by means of nutrient intake. As expected, our work revealed that dietary protein restriction induced skeletal muscle exhibited higher expression of factors related to slow-twitch muscle and lower expression of factors related to fast-twitch muscle than a normal protein diet. Similarly, the expression trends of *MYH4* and *MYH7* were the same as those of fast and slow muscles in skeletal muscle in weaned piglets, respectively. In our current study, we also observed that pigs offered a lower protein diet showed elevated SDH activity associated with oxidative muscle fibers and decreased LDH activity and LA content associated with glycolytic muscle fibers in skeletal muscle, which was similar to the findings of previous study.[4,5] Salvatore et al. reported that thyroid hormones play a role in driving the transcription of fast-twitch muscle fiber-related genes, such as MYH1, MYH2, and MYH4.[27] In addition, T3 reduces the expression and activity of downstream transcription factors involved in maintaining the slow-twitch phenotype of muscle fibers,[28] suggesting that thyroid hormones are potent regulators of muscle fiber phenotypes.[27] Indeed, we found that dietary protein restriction led to a substantial reduction in serum T3 levels and T3/T4 in weaned piglets, which was also associated with the phenotypes of muscle fibers. Notably, in the present study, dietary protein restriction significantly decreased skeletal muscle weights in weaned piglets. This may be attributed to the reduction in dietary protein content inhibiting muscle collagen production, promoting collagen degradation and decreasing muscle hardness,[29] and may also be related to changes in the types of muscle fibers.[30] Together, these findings reveal that a dietary protein restriction strategy is an effective method of skeletal muscle fiber type regulation.

Accumulating amounts of evidence have shown that skeletal muscle development depends on the differentiation of myogenic progenitors, which are regulated by the expression of myogenic regulatory factors (MRFs), such as *Pax3*, *Pax7*, *Desmin*, *Myf5*, *MyoD*, *Myogenin*, and *Myoglobin*.[31–35] In the present work, a significant difference in expression of *Pax3*, *Pax7*, *Desmin*, *Myf5*, *MyoD*, *Myogenin*, and *Myoglobin* was observed for pigs under dietary protein restriction, showing that dietary protein restriction mediated the expression of these genes to coordinate metabolism and development in skeletal muscle. Among these genes, *Pax3* and *Pax7* are critical genes that regulate early muscle development and induce myogenic differentiation.[31] Failure to express *Pax3* or *Pax7* leads to developmental obstruction.[31] *Desmin* is an indicator of postmortem protein degradation associated with skeletal muscle fiber type, and the results revealed that type IIb muscle fibers showed faster degradation velocity of *Desmin* than type I fibers.[32] Notably, our study suggests that pigs undergoing dietary protein restriction exhibit higher mRNA expression of *Pax3*, *Pax7*, *Desmin*, *Myf5*, *Myogenin*, and *Myoglobin* and the lower mRNA expression of *MyoD* than pigs fed normal dietary protein. Recent study results have shown that *MyoD* is a critical transcriptional regulatory factor for fast muscle fibers in mice.[33] This observation also reflects that the expression trend of *MyoD* in our study was associated with the transformation of muscle fiber type induced by dietary protein restriction. Furthermore, as an important indicator of the abundance of slow-twitch muscle fibers, the gene and protein expression of Myoglobin was also higher in skeletal muscle of piglets treated with dietary protein restriction.[36] Taken together, these results indicate that the muscle fiber type transformation induced by dietary protein restriction may be one of the reasons for the change in MRFs.

Different FGFs have been shown to exert varied roles in muscle in response to dynamic physiological environments. It has been proven that FGF19 regulates skeletal muscle mass and protects against muscle atrophy by increasing muscle fiber size by stimulating the phosphorylation of ERK1/2 and mTOR pathway-associated proteins.[37] Similarly, concerning FGF21, previous work has found that FGF21 contributes to protecting against muscle weakness and improving skeletal muscle metabolism.[8,38] More importantly, the protein restriction pathway described

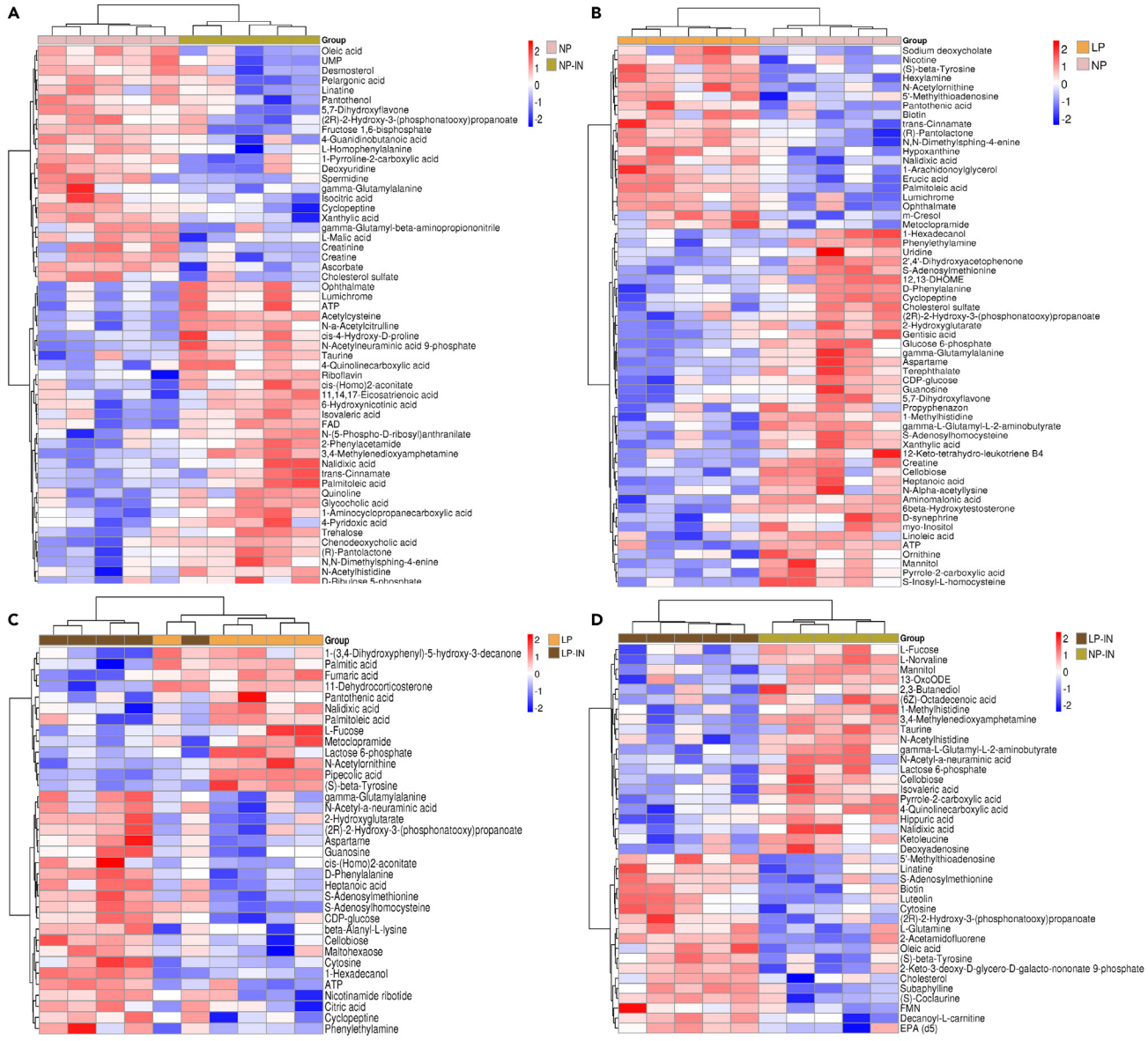

Figure 7. Hierarchical clustering heatmap of differential metabolites (DM)

(A) Heatmap of NP vs. NP-IN.

(B) Heatmap of LP vs. NP.

(C) Heatmap of LP vs. LP-IN.

(D) Heatmap of LP-IN vs. NP-IN. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. n = 5.

in many studies was also found in our results, for example, decreased diet protein significantly increased FGF21 expression in skeletal muscle in pigs, suggesting that FGF21 is a critically essential dietary protein restriction-sensing factor.[24,25] Notably, a previous report suggested that the ERK1/2 signaling pathway is governed by FGF21.[39] In keeping with these data, our studies also observed that dietary protein restriction-induced FGF21 expression elevation significantly activates the phosphorylation of ERK1/2 signaling in skeletal muscle in weaned piglets. ERK1/2 signaling is for maintaining skeletal muscle homeostasis, promoting MHC expression, which mediates fiber type conversion to a slow oxidative phenotype with type I MHC expression.[14,40] Moreover, studies have shown that ERK1/2 signaling plays a key regulatory role in skeletal muscle hypertrophy, muscle cell fusion, and myogenic differentiation.[37,41–43] In this study, our findings demonstrated that ERK1/2 inhibitor administration significantly blocked the phosphorylation of ERK1/2 and the ratio of phosphorylated ERK1/2 to ERK1/2, regardless of dietary protein levels. Blocking ERK1/2 signaling can eliminate skeletal muscle fiber type switching in weaned piglets. This

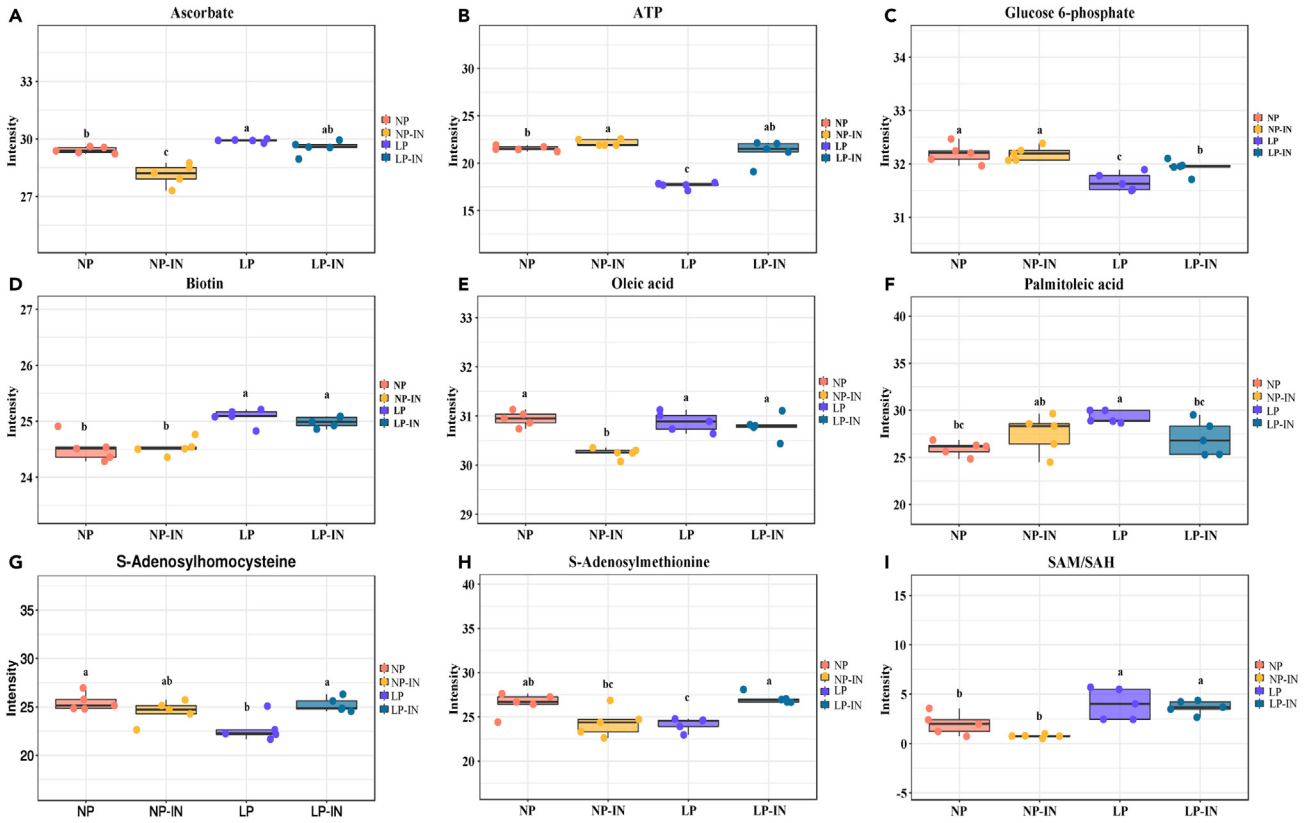

Figure 8. Representative DM in skeletal muscle of weaned piglets with dietary protein restriction and ERK1/2 inhibitor administration

The abundance of ascorbate (A), ATP (B), glucose-6-phosphate (C), biotin (D), oleic acid (E), palmitoleic acid (F), SAH (G), SAM (H), and SAM/SAH (I) in skeletal muscle. n = 5. One-way analysis of variance (ANOVA) was performed to analyze representative DM. NP = normal protein diet, NP + IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor. Data are presented as the mean ± SEM. Bars with different letters are defined as significant at $p < 0.05$.

may imply that ERK1/2 signaling is necessary for dietary protein restriction to improve muscle fiber properties. Furthermore, our work also showed that ERK1/2 signaling inhibition led to an increase of *MyoD* expression and decreases of *Pax3*, *Pax7*, *Desmin*, *Myf5*, *Myogenin*, and *Myoglobin* expression. Overall, these findings suggest that ERK1/2 signaling alters the expression of genes related to myogenic differentiation, which may partly account for the remodeling of skeletal muscle fibers.

mTORC1 is a ternary complex composed of mTOR, mTOR regulatory protein (Raptor), and mammalian lethal with SEC13 protein 8 (mLST8).[44] mTORC1 systematically coordinates metabolism through different signaling pathways to improve anabolism and cell growth while inhibiting catabolism, such as nutrient intake (GTPases RAG-A, RAG-B, RAG-C, and RAG-D), regulation of growth factor stimulation (PI3K-AKT-TSC signaling), and perception of cellular energy state (AMP-activated protein kinase).[44] Furthermore, mTORC1 signaling can be activated in different tissues, although the mechanism varies. In muscle satellite cells, Pax3 can significantly induce mTORC1 signaling to protect cells from external stimulation.[35] On the other hand, HEK293 cells exhibit higher expression of ERK1/2 phosphorylation to hyperactivate mTORC1 signaling.[45] In this study, the results showed that dietary protein restriction-induced skeletal muscle presented higher levels of ERK1/2 phosphorylation, which hyperactivated mTORC1 signaling in weaned piglets. Activated mTORC1 stimulates the expression of PGC-1α, which promotes slow-twitch muscle fiber generation.[36] However, obstruction of mTOR signaling reduces the expression of the mitochondrial transcription regulator PGC-1α, leading to decreased mitochondrial gene expression and oxygen consumption in skeletal muscle tissue and cells.[46] In our present study, the hyperactivation of ERK1/2-mTORC1 signaling and muscle fiber type switching were abolished by normal protein intake or ERK1/2 inhibitor treatment in skeletal muscle in weaned piglets. Therefore, dietary protein restriction may be involved in the transformation of muscle fiber type through the combined effect of FGF21-mediated ERK1/2 activation and the cascade activation of mTORC1/PGC-1α signaling.

Metabolomic analysis of skeletal muscle revealed a relationship between dietary protein levels and muscle fiber type switching in weaned piglets treated with both dietary protein restriction and ERK1/2 inhibitor. Glucose-6-phosphate and ATP content are critical indicators of muscle glycolytic ability after slaughter.[47] Studies have shown that skeletal muscle rich in type IIb muscle fibers contains more glycogen and has greater glycolytic potential to produce lactic acid and energy than muscles with a higher proportion of oxidative muscle fibers.[47] In this study, our data suggest that dietary protein restriction decreases ATP and glucose-6-phosphate content in skeletal muscle in weaned piglets.

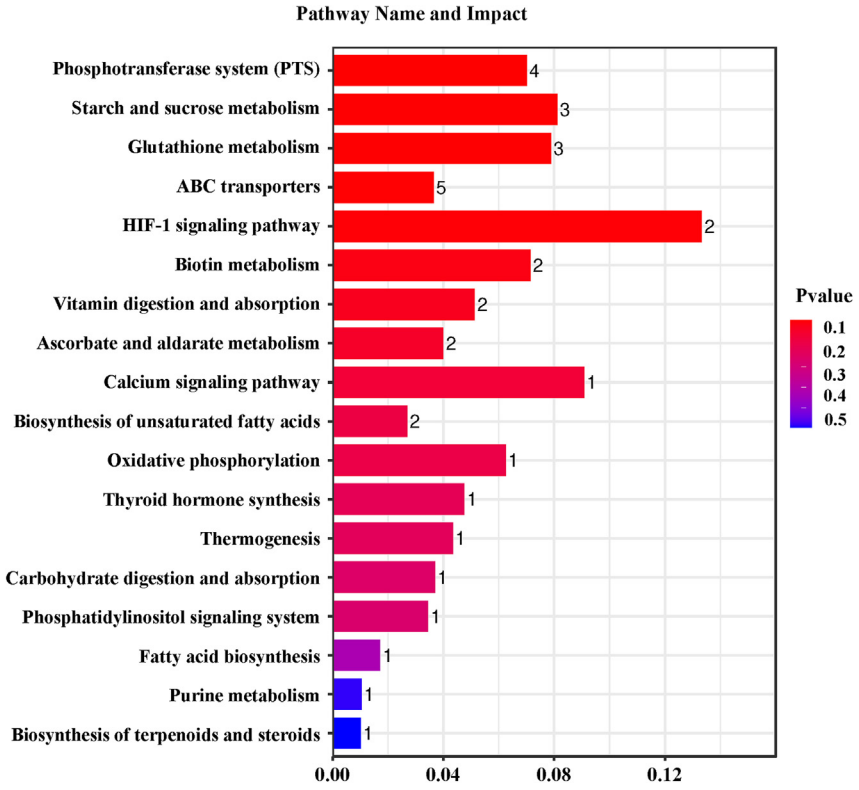

Figure 9. Enrichment of DM in KEGG pathways in the NP-IN vs. NP vs. LP-IN vs. LP groups

The vertical coordinate represents the metabolic pathway, and the horizontal coordinate represents the Impact value enriched into different metabolic pathways, which can be understood as the contribution degree, that is, the higher the value, the higher the contribution degree of metabolites detected in this pathway. The color is correlated with the p-value. The redder the color, the smaller the p-value, and the bluer the color, the larger the p-value. The smaller the p-value, the more significant the detected differential metabolites have on the pathway.

Moreover, we found that ERK1/2 inhibitor increased the content of ATP and glucose-6-phosphate in skeletal muscle under dietary protein restriction. These results also reflect that dietary protein restriction and ERK1/2 inhibitors may significantly affect skeletal muscle fiber type and muscle metabolites. On the other hand, intramuscular fat metabolism is associated with skeletal muscle fatty acid metabolism. Increased quantities of palmitoleic acid were observed in our present work in skeletal muscle under dietary protein restriction and may be related to ERK1/2 signaling.[48] In C2C12 cells, oleic acid significantly increased the proportion of type I muscle fibers, a result similar to our findings, which showed that the ERK1/2 inhibitor significantly reduced the oleic acid content in the skeletal muscle of a normal protein diet, accompanied by a decrease in the proportion of type I muscle fibers.[49] In addition, our results show that a low protein diet-induced elevation in biotin and ascorbate significantly increased muscle antioxidant capacity and promoted muscle development, respectively.[50,51] Furthermore, recent work reported that SAM and SAH are a crucial methyl group donor and methyltransferase inhibitor, respectively, and the SAM/SAH ratio can reflect the degree of methylation to a certain extent.[52] Interestingly, high methyltransferase levels and high methylation levels are beneficial to promote the formation of red muscle in fish.[53] In addition, mTORC1 signaling activation is also associated with increased SAM levels due to high levels of methionine.[44] In this work, our findings showed that dietary protein restriction-induced skeletal muscle showed higher levels of SAM/SAH, which may promote slow-twitch muscle fiber generation in weaned piglets. Importantly, the significant KEGG pathways for meat quality regulation were identified to be the HIF-1 signaling pathway, calcium signaling pathway, biotin metabolism, biosynthesis of unsaturated fatty acids and oxidative phosphorylation. Previous studies have revealed that these signaling pathways are involved in the regulation of meat quality, such as the HIF-1 signaling pathway, which is activated under hypoxic conditions to regulate glycolysis.[54–56] In addition, calcium signaling pathways are closely related to meat quality can mediate the transformation of skeletal muscle fibers from fast-twitch muscle fiber to slow-twitch muscle fiber and improve intramuscular fat deposition.[57–59] Overall, these results imply that intricate mechanisms may be involved in the causal relationship between meat quality regulation and dietary protein restriction, and ERK1/2 signaling might mediate muscle fiber remodeling in different ways under various conditions.

In conclusion, we demonstrated that dietary protein restriction induced muscle fiber type switches from fast-twitch muscle fiber to slow-twitch muscle fiber association the regulation of FGF21-ERK1/2-mTORC1 signaling in skeletal muscle in weaned piglets. Multiple *in vivo* evidence suggested that pigs offered a protein restriction diet had significantly altered metabolic enzyme activities and abundance of metabolites associated with skeletal muscle type remodeling. However, the blockade of ERK1/2 signaling significantly attenuated the conversion

effect from fast-twitch muscle to slow-twitch muscle and altered the corresponding skeletal muscle fiber properties and metabolites in weaned piglets. Our work contributes to better identifying the underlying mechanism by which dietary protein restriction improves meat quality in mammals, which provides a theoretical basis for the application of dietary protein restriction.

Limitations of the study

The findings of this study have to be seen in light of some limitations. We identified potential genes by which dietary protein restriction improves meat quality by investigating the effect of dietary protein restriction on muscle fiber development in weaned piglets, and highlighted the regulatory mechanisms. However, the results of muscle fiber type switching remain to be verified in fattening pigs and *in vitro* trials.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, S.L., T.D.Z., and J.M.Y.; Methodology, S.L. and T.D.Z.; Investigation, S.L., H.P.Z., and Z.Y.H.; Resources, T.D.Z. and J.M.Y.; Data curation, S.L. and J.M.Y.; Writing–original draft, S.L.; Writing–Review and editing, S.L., Z.R.W., and J.M.Y.; Visualization, S.L. and J.C.; Supervision, T.D.Z., and J.M.Y.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FGF21 antibody	Abcam	Cat # ab171941; RRID: AB_2629460
Anti-Slow MyHC antibody	Abcam	Cat # ab11083; RRID: AB_297734
Anti-Fast MyHC antibody	Abcam	Cat # ab91506; RRID: AB_10714690
Anti-ERK1/2 antibody	Cell Signaling Technology	Cat # 9102; RRID: AB_330744
Anti- Phospho-ERK1/2 antibody	Cell Signaling Technology	Cat # 9101; RRID: AB_331646
Anti-mTOR antibody	Cell Signaling Technology	Cat # 2972; RRID: AB_330978
Anti-Phospho-mTOR antibody	Cell Signaling Technology	Cat # 2971; RRID: AB_330970
Anti-S6 antibody	Cell Signaling Technology	Cat # 2217; RRID: AB_331355
Anti-Phospho-S6 antibody	Cell Signaling Technology	Cat # 4858; RRID: AB_916156
Anti-Raptor antibody	Santa Cruz Biotechnology	Cat # SC-81537; RRID: AB_2130791
Anti-Phospho- Raptor antibody	Signalway Antibody	Cat # 12778; RRID:AB_3094614
Anti-Myoglobin antibody	Proteintech	Cat # 16048-1-AP; RRID: AB_1640050
Anti-PGC1a antibody	Proteintech	Cat # 66369-1-Ig; RRID: AB_2828002
Anti-Beta Actin antibody	Proteintech	Cat # 66009-1-Ig; RRID: AB_2687938
Goat Anti-Mouse IgG(H+L)	Proteintech	Cat # SA00001-1; RRID: AB_2722565
Goat Anti-Rabbit IgG(H+L)	Proteintech	Cat # SA00001-2; RRID: AB_2722564
Chemicals, peptides, and recombinant proteins		
ERK1/2 Signaling Inhibitor	Selleck	Cat # S1020
Critical commercial assays		
Triiodothyronine Assay Kit	Nanjing Jiancheng Bioengineering Institute	Cat # H222-1-2
Thyroxine	Nanjing Jiancheng Bioengineering Institute	Cat # H223-1-2
Creatine Kinase assay kit	Nanjing Jiancheng Bioengineering Institute	Cat # A032-1-1
Succinate Dehydrogenase assay kit	Nanjing Jiancheng Bioengineering Institute	Cat # A022-1-1
Lactate Dehydrogenase assay kit	Nanjing Jiancheng Bioengineering Institute	Cat # A020-2-2
Lactic Acid assay kit	Nanjing Jiancheng Bioengineering Institute	Cat # A019-2-1
Total Protein Extraction Kit	Solarbio	Cat # BC3790
TransZol Up Plus RNA Kit	TransGen Biotech	Cat # ER501-01-V2
EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix	TransGen Biotech	Cat # AE311-02
Deposited data		
Muscle metabolomic data	This study	The EMBL-EBI MetaboLights database with the identifier MTBLS9540
Experimental models: Organisms/strains		
Pig: Landrace Yorkshire weaned piglets	Jiangxi Aoyun Agricultural Development Co. LTD	This paper
Oligonucleotides		
See Table S3 for primers for qRT-PCR	N/A	This paper
Software and algorithms		
GraphPad Prism	GraphPad	https://www.graphpad.com/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SPSS 24.0 software	SPSS	https://www.ibm.com/analytics/spssstatistics-software
Proteo Wizard software	Smith et al.[60] and Navarro-Reig et al.[61]	https://proteowizard.sourceforge.io/
Ropls software	Thevenot et al.[62]	https://bioconductor.org/packages/release/bioc/html/ropls.html
Image-Pro Plus	Media Cybernetics	https://mediacy.com/image-pro/
Other		
See Table S2 for ingredients and nutrient levels of the basal diets for weaned pigs	N/A	This paper

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jinming You (youjinm@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- All untargeted metabolomic data used in this publication have been deposited to the EMBL-EBI MetaboLights database with the identifier MTBLS9540. The complete dataset can be accessed at https://www.ebi.ac.uk/metabolights/MTBLS9540.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal model

The experiment was carried out from November 2021 to December 2021 in the Pig Nutritional Metabolism Test Base of Jiangxi Key Innovation Center of Production and Education Integration of Quality and Safety Livestock and Poultry Production, Jiangxi Agricultural University. All animal experimental procedures used in this study were reviewed and approved by the Animal Care and Use Committee of Jiangxi Agricultural University (JXAULL-2021-035). A three-day pre-feeding with twenty-four 21-day-old male Landrace Yorkshire weaned piglets purchased from a commercial farm (Jiangxi Aoyun Agricultural Development Co. LTD, Nanchang, China) was performed, and the pigs were randomly divided into two groups according to body weight (7.25 $\pm$ 0.12 kg), and were fed a normal CP diet (19.78% CP, which met the nutritional requirements of weaned piglets recommended by the National Research Council 2012) or a low CP diet (16.91% CP) (Table S2). The dietary CP content was changed by adjusting the amount of soybean meal and puffed corn in the formula. Compared with the normal CP (NP) diet, the low CP (LP) diet contained higher levels of puffed corn and lower levels of soybean meal (Table S2). The supplemental amino acids (lysine, methionine, threonine, tryptophan, valine, leucine and isoleucine) were added to meet the pigs' requirements and maintain amino acid balance among groups. Both feeds were powdered. Next, half of the pigs in each group were randomly selected to receive weekly intraperitoneal injections of 0.5 mg/kg ERK1/2 signaling inhibitor (PD184352, Selleck, Houston, USA), and the rest were injected with an equivalent dose of the vehicle (Dimethyl sulfoxide), which constituted 4 treatment groups (n = 6, NP = normal protein diet, NP+IN = normal protein diet + ERK1/2 signaling inhibitor, LP = low protein diet, LP + IN = low protein diet + ERK1/2 signaling inhibitor). Previous studies have shown that injection of 0.1-1mg/kg ERK inhibitor can effectively inhibit ERK1/2 signaling in newborn piglets. Therefore, we chose an average injection dose of 0.5mg/kg. Moreover, we conducted a pilot experiment demonstrating that this dose can effectively block ERK1/2 signaling in weaned piglets. In addition, according to a published formulation, this dose is equivalent to mice consuming about 3mg/kg of the ERK1/2 inhibitor and successfully inhibiting ERK1/2 signaling.[60–66] All pigs were housed in single cages and had free access to food and water during the 28 days of the experiment. The health statuses of the piglets were observed and recorded regularly to minimize the stress of pigs.

METHOD DETAILS

Sample collection

At the end of the experiments, blood samples were collected via the vena cava anterior and centrifuged at 4°C to collect serum and were stored at -80°C for further analysis (T3 and T4). All pigs were sacrificed quickly by bloodletting to collect muscle samples. The semitendinosus

was collected accurately and completely along the dividing line between each muscle part. After weighing, the fresh skeletal muscle was divided into two parts: one was cut into cuboids and placed in a special perforated tube for the preparation of frozen sections,[67] and the other section was frozen in liquid nitrogen and transferred to the -80°C freezer for further analysis. The semitendinosus muscle is one of the skeletal muscles closely related to meat quality. Therefore, it is of great significance to elucidate the composition and transformation mechanism of muscle fiber types of semitendinosus muscle for the regulation of meat quality.[68,69]

Determination of serum T3 and T4

Serum T3 and T4 levels were measured using commercial assay kits (H222-1-2, H223-1-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Refer to the kit instructions for the test operation. The inter- and intra-CV were < 12% and < 10%, respectively.

Histological analysis

The skeletal muscle samples were removed from liquid nitrogen and placed in a freezing microtome (CM1850, Leica, Wetzlar, Germany). After the temperature stabilized, the tissue samples were fixed and sliced continuously with a thickness of 6 μm. The staining process of muscle fibers includes acid incubation, washing, neutral dyeing, washing, alkaline incubation, washing, dyeing, dehydration and sealing.[67] Based on myosin adenosine triphosphatase (ATPase) activities, the muscle fiber category was classified into type I, IIa, and IIb. The color of type I fiber is the darkest, type IIb fiber is the lightest, and type IIa fiber is the center.[70] Three visible fields without freezing damage or tissue damage were chosen randomly for each sample. Image software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, MD, USA) was used to calculate the total number of muscle fibers in each sample and the number of different muscle fiber types. Fiber density (fiber number/mm^2) was calculated by the mean number of muscle fibers per mm^2.

Measurement of muscle metabolic enzyme activities

One hundred milligrams of muscle tissue were weighed and rinsed in precooled 0.9% NaCl to remove blood. The muscle and 0.9% NaCl were placed in a certain proportion (the total volume of 0.9% NaCl was 9 times the weight of the tissue) in the EP tube, and the homogenate grinding beads were added. The prepared tissue homogenates were centrifuged for 10 min at 4 °C and 2000 r/min, and the supernatant was taken for further analysis. The activities of SDH, creatine kinase (CK), LDH and lactic acid (LA) content were measured with commercial assay kits (A022-1-1, A032-1-1, A020-2-2 and A019-2-1. Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Refer to the kit instructions for the test operation.

Western blotting

Protein extracts from skeletal muscle were isolated by a Total Protein Extraction Kit (BC3790, Solarbio, Beijing, China). After 7.5%, 10% and 15% SDS-PAGE, the proteins were transferred to PVDF membrane. The membranes were blocked with 5% milk and then probed with specific antibodies.[71] Primary antibodies FGF21, slow-MyHC and fast-MyHC were purchased from Abcam (ab171941, ab11083 and ab91506, Cambridge Science Park, UK). ERK1/2, p-ERK1/2, mTOR, p-mTOR, S6 and p-S6 antibodies were obtained from Cell Signaling Technology (9102S, 9101S, 2972S, 2971S, 2217S and 4858S, Danvers, MA, USA). Raptor antibody was obtained from Santa Cruz (sc-81537, Santa Cruz Biotechnology, Inc. Santa Cruz, USA). p-Raptor antibody was obtained from Signalway Antibody (12778-2, Maryland, USA), β-actin, Myoglobin, PGC-1a, and HRP-linked secondary antibodies were obtained from Proteintech (81115-1-RR, 16048-1-AP, 66369-1-Ig, SA00001-1 and SA00001-2, Wuhan, China). The dilution ratios of the primary and secondary antibodies were 1:1000 and 1:5000, respectively.

Quantitative real-time PCR analysis

The total RNA of skeletal muscle was extracted by a commercial kit (TransZol Up Plus RNA Kit, TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Then, the RNA was reverse-transcribed into cDNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Quantitative real-time PCR was processed on a CFX96 RT-PCR Detection System (Bio-Rad).[71] Target gene mRNA expression and standardization methods refer to use the method of $2^{-\Delta\Delta CT}$.[72] Primer sequence details are presented in Table S3.

Muscle metabolomics analysis

Muscle samples were fully ground, lysed and centrifuged, the supernatant was added to acetonitrile solution, and the filtrate was obtained for liquid chromatography–mass spectrum (LC–MS) analysis.[73] The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150 × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column temperature was maintained at 40°C. The flow rate and injection volume of the system were 0.25 mL/min and 2 μL, respectively. For the analysis of LC-ESI (+)-MS, the mobile phases were prepared of (A2) 0.1% formic acid in water (v/v) and (B2) 0.1% formic acid in acetonitrile (v/v). The separation process was conducted in the gradient shown as follows: 0∼1 min, 2% B2; 1∼9 min, 2%∼50% B2; 9∼12 min, 50%∼98% B2; 12∼13.5 min, 98% B2; 13.5∼14 min, 98%∼2% B2; 14∼20 min, 2% B2. Moreover, the (B3) acetonitrile and (A3) ammonium formate (5mM) was selected to do the LC-ESI (-)-MS analysis. The separation was conducted in the gradient shown as follows: 0∼1 min, 2% B3; 1∼9 min, 2%∼50% B3; 9∼12 min, 50%∼98% B3; 12∼13.5 min, 98% B3; 13.5∼14 min, 98%∼2% B3; 14∼17 min, 2% B3.[74] Mass spectrometric

detection of metabolites was conducted on an Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used.[75]

QUANTIFICATION AND STATISTICAL ANALYSIS

The raw data were first converted to mzXML format by MS Convert in the Proteo Wizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment.[76,77] Ropls software was used for all multivariate data analyses and modeling.[78] After scaling the data, models were built on principal component analysis (PCA) and partial least-square discriminant analysis (OPLS-DA), which showed the differences in metabolite composition between samples. The overfitting test of the model was performed by the permutation test. OPLS-DA allowed for the determination of discriminating metabolites using the variable importance on projection (VIP). The *P* value, VIP value produced by OPLS-DA, and fold change (FC) were applied to discover the contributable-variable for classification. $P < 0.05$ and VIP > 1 were considered statistically significant metabolites.

Data analysis for serum T3 and T4 concentrations, muscle SDH, CK and LDH activities, muscle LA content, muscle weight, muscle fiber parameter, muscle factor mRNA and protein expression, were conducted using two-factor analysis of variance, and Duncan's method was used to make multiple comparisons between each group (version 24.0, SPSS Institute, Inc., Chicago, IL). GraphPad Prism 9.0 was used to generate the graphs. The results are presented with as the mean values with standard error (SEM), and bars with different letters are defined as significant at $P < 0.05$. All the statistical details of the experiment can be found in the figure legend.