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FNDC5 IS THE KEY MOLECULE TO INHIBIT MUSCLE FIBER DEVELOPMENT IN TIBETAN PIGS

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ABSTRACT

Differences in muscle fiber development between Tibetan and Yorkshire pigs determine their performance in terms of meat quality, taste, and food value. To investigate the molecular regulation of muscle fiber development by fibronectin type III domain-containing protein 5 (*FNDC5*), a preliminary study of *FNDC5* gene expression in the longissimus dorsi muscle and Leg muscle tissues of Tibetan and Yorkshire Pigs was conducted using RT–qPCR and western blotting. Muscle tissues were sectioned and stained to observe the muscle fiber diameter and area under a microscope. The mRNA and protein expression levels of *FNDC5*, and the diameter and area of muscle fibers in the longissimus dorsi muscle and Leg muscle tissues of Tibetan pigs were significantly lower than those in Yorkshire Pigs. The results imply that *FNDC5* negatively regulates muscle fiber diameter in pigs. The role of *FNDC5* in pork quality has important implications for improving pork production efficiency, pork quality, and the sustainable development of the livestock industry.

Keywords: Pig; *FNDC5*; Gene expression.

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INTRODUCTION

Skeletal muscle development is a highly complex and closely coordinated process(Buckingham and Rigby 2014). In mammals, skeletal muscle is a heterogeneous mesodermal structure that forms on both sides of the neural tube and accounts for a large part of the organism(Bentzinger, Wang, and Rudnicki 2012). According to the myosin composition, skeletal muscle fibers can be divided into Myh1, Myh2, Myh4, and Myh7(Schiaffino and Reggiani 2011). Muscles mainly composed of Myh2 are classified as fast-oxidizing muscle fibers, between oxidizing muscle fibers (Myh7) and glycolytic muscle fibers (Myh4). Thus, Myh2 provides energy to the body under both aerobic and anaerobic conditions(Mancini and Hunt 2005). Fibronectin type III domain-containing protein 5 (FNDC5) is a transmembrane protein composed of a C-terminal hydrophobic structure, signal peptide, and fibronectin domain.(Schumacher *et al*. 2013) It was first identified in 2002 and is expressed in skeletal muscle, heart, and brain(Teufel *et al*. 2002; Ferrer-Martínez, Ruiz-Lozano, and Chien 2002; Boström *et al*. 2012) However, most of the functions of *FNDC5* were not understood until 2012, when it was found that there is an inextricable regulatory mechanism between its cleavage to produce irisin and *FNDC5*(Brenmoehl *et al*. 2014), and may mediate some of the beneficial effects of exercise(Lavi *et al*. 2022; Boström *et al*. 2012; Jedrychowski *et al*. 2015; Wrann *et al*. 2013) The expression of *FNDC5* in different organ tissues of different livestock has been reported.(Hofmann, Elbelt, and Stengel 2014) It affects muscle metabolism by regulating lactate production, fat oxidation, and mitochondrial biosynthesis(Lavi *et al*. 2022), and promotes fat-to-muscle conversion and weight loss(Boström *et al*. 2012). However, whether *FNDC5* regulates the development of muscle fibers has been rarely reported, and the role of this gene in pork quality is currently unclear.

Pork is the most important source of dietary protein for humans, and the development and growth of skeletal muscles determine muscle yield and quality(Asp *et al*. 2011). The basic unit of muscle tissue is muscle fiber, which is also an important factor in the response of muscle properties. Three indicators reflect the physiological properties of muscle fibers: the number, type, and diameter. The diameter and number of muscle fibers determine the muscle yield(Glass 2003) and can have an important influence on meat quality indicators, including tenderness, juiciness, and tethering power(Choe *et al*. 2008; Ryu and Kim 2005; Ryu, Choi, and Kim

2005) As such, they have become a research focus.(Lawrie 1970)

Tibetan and Yorkshire Pigs are the two main pig breeds on the Qinghai–Tibet Plateau, which is the largest high-altitude ecosystem in the world.(Yang *et al*. 2011) Tibetan pigs occupy an important position in the plateau region and provide a sustainable natural resource for the selection and development of pig breeds. Moreover, Tibetan pigs are small in size, and their similarity to humans renders them an excellent model for human research. As such, it is of great economic value to carry out research related to Tibetan pigs. Yorkshire Pigs are raised on a large scale around the world because of their rapid growth rate, high feed conversion rate, high leanness, and good meat color (Brenmoehl *et al*. 2014) .Tibetan pigs have more mitochondria and myoglobin in their muscle fibers, which help them to survive in low-oxygen, food-scare environments; in contrast, Yorkshire Pigs have more myosin in their muscle fibers, which makes them more suitable for rapid movement and growth.(Gan *et al*. 2019). The longissimus dorsi muscle of Tibetan pigs contain higher levels of Myh2, Leucine, Valine, and Isoleucine than those of Yorkshire Pigs, and there are major differences in muscle quality between Tibetan and Yorkshire Pigs(Gan *et al*. 2019)

To further investigate the expression of *FNDC5* at the muscle level, Tibetan and Yorkshire Pigs were selected for analysis. The expression of *FNDC5* in the longissimus dorsi muscle and Leg muscle tissues at 30, 90, and 180 days of age was analyzed by real-time PCR(RT–qPCR) and western blotting. Muscle sections were collected from 180-day-old pigs to analyze the diameter, area, and number of skeletal muscle fibers. The expression patterns of *FNDC5* in different breeds and growth stages were interpreted and elucidated. This study provides a new reference for the elucidation of the regulatory mechanisms of *FNDC5* in muscle tissue. The results provide a reference for analysis of the molecular mechanisms of pork quality and for improving meat processing quality.

MATERIALS AND METHODS

Sample collection: In this experiment, Tibetan and Yorkshire Pigs from the Nyingchi region of Tibet were selected as experimental animals. Both breeds were in the same living environment and underwent the same feeding and immunization processes (both breeds were depopulated boars); however, the breeds were not related to each other. A total of 10 30-, 90-, and 180-day-old Tibetan and Yorkshire Pigs were slaughtered, and their longissimus dorsi muscle and Leg muscle tissues were collected. Then, 8–10 g of tissue was cut, inserted in sampling tubes with RNA preservation solution and blank, and immediately placed in liquid nitrogen; 3 × 6 cm pieces of longissimus dorsi muscle tissue were placed in sampling tubes with formalin, sealed with sealing film, and stored at room temperature. All procedures were performed in strict accordance with the program approved by the Animal Welfare Committee of the Tibetan Agricultural and Animal Husbandry University (license number: XK622).

Total RNA extraction and cDNA synthesis: Based on previous studies, RNA was extracted from muscle tissue using the TRIzol method and subsequently solubilized with RNase-free ddH_2 O to a suitable concentration and stored at −80 °C for backup (Gancz and Gilboa 2017). RNA samples were assessed for DNA and protein contamination using 1% agarose gel electrophoresis, with subsequent concentration and quality analysis performed via micro-spectrophotometry using Nano Drop 2000, and a portion was stored at −20 °C for backup. The total RNA was reverse transcribed using a one-step cDNA reverse transcription kit (Beijing Tiangen Biochemical Technology Co., Ltd., KR180123), and stored at −20 ℃. Selection of β-actin as an internal reference gene (Table 1),A pair of sequence-specific primers for the coding region of *FNDC5* (Table 1) was designed using Primer premier 5.0 software to amplify the coding region based on the mRNA sequence of wild boar *FNDC5* published in GenBank on the NCBI website (accession number XM_021095832.1) and synthesized by Biotech Biological Co. TE (Trypsin-EDTA Solution) was solubilized and stored at 4 ℃.

Table 1. Primer sequences used to amplify the coding region of *FNDC5*.

Primer name	Sequences	Product size	Annealing temperature
FNDC5	F:GGTTGTCATCGGATTGCC R:TCCTCCTCCAGGTCCCAGA	157 bp	60 °C
β-actin	F: TCTGGCACCACACCTTCTA R:AAGGTCTCGAACATGATCTG	127 bp	60 °C

RT–qPCR: The cDNA of the qualified longissimus dorsi muscle and Leg muscle were selected as templates for fluorescent quantitative PCR assays. Standards were set (1 μL from all samples, mixed well), and three replicates of each sample were prepared. The total volume of the PCR reaction is 20 μL, including 10 μL of 2×SuperReal

PreMix Plus, 0.5 mL (10 mmol/L) forward and reverse primers (Table 1), and 2 mL DNA template, and RNase-free ddH_2O to a total volume of 20 µL. The PCR reaction procedure was as follows: pre-denaturation at 95 °C for 10 min; denaturation at 95 °C for 30 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s, for a total of 40 cycles. The Ct value was used to calculate the expression of mRNA, which was plotted and analyzed with $2^{-\Delta\Delta Ct}$ values.

Western blotting: The extracted proteins were used as an internal reference for Lamin B1; the concentration was determined by the BCA method and the sample volume was calculated: Configure the BSA standard according to the instructions of BCA protein quantification kit, the concentrations were 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0. Take out the 96-well enzyme labelling plate, and add 25 µL of standard to each well, and do 3 replicates for each concentration. Take 5 µL of protein sample and mix it with 95 µL of saline, make a 5-fold dilution of the target protein, add 25 µL of standard per well, and make 3 replicates for each sample. Make BCA working solution by mixing solution A and solution B at the ratio of 50:1, add 200 µL of working solution into each well, put it into the enzyme marker and shake it for 1 minute, incubate it at 37 ℃ for 30 minutes. After that, the absorbance was measured at 562 nm with shaking for 1 minute and the concentration of protein was calculated.The proteins were separated by electrophoresis, and the target gel was cut and transferred onto a PVDF membrane. The gel was blocked with 5% skim milk powder at room temperature for 1 h. The primary antibody was incubated at 4 °C overnight, and the membrane was washed three times with TBST for 10 min/time; the secondary antibody was incubated at 37 °C for 1 h, and the membrane was washed again with TBST for three times for 10 min; the target bands were visualized by ECL color development.

HE staining: The collected muscle tissues were removed from formalin for treatment and soaked in running tap water for 24 h. The tissues were then soaked in different concentrations of alcohol for dehydration, soaked in xylene solution I and xylene solution II for 30 min to make them transparent, and then embedded in wax for 3 h. Sections were obtained after the embedded tissue wax blocks had thoroughly solidified. The sections were first rehydrated with different concentrations of alcohol, placed in eosin staining for 10 min, followed by rinsing under running water for 5 min, then placed in hematoxylin staining solution for 5 min. Next, they were rinsed under running water for 5 min and then by differentiation solution for 3–5 s, placed in a basin of water for 5 min, and repeated three times. Finally, the sections were re-blued with a re-blueing solution for 10 s and rinsed under running water. Stained sections were soaked in various concentrations of alcohol and xylene. After the above steps were completed, the sections were sealed with neutral resin, blow-dried using a fan, and examined under a microscope.

Indicator measurement: One section of the longest dorsal muscle sample from each pig of Tibetan and Yorkshire pig, 3 heads of each breed were selected, and 3 fields of view with clear staining of muscle fibres were randomly selected in the section, in order to avoid errors caused by human selection, the 3 fields of view pictures were evenly distributed in the section. Data measurements were performed with image analysis software (Image-Pro Plus 6.0).

(1) Muscle fiber diameter

A unit area of visual field was taken under the field of view with a self-contained measuring tape, and the long diameter as well as the short diameter of the muscle fibres were measured in accordance with the principle of counting up but not down, counting left but not right, and taking the average of the long and short diameters, d=(long diameter + short diameter)/2, with d being the average value of the diameter of the muscle fibres in the unit area.

(2) Muscle fiber area

The cross-sectional area of the muscle fibres was measured according to the principle of counting up, not down, and counting left, not right.

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA) and correlation regression using the SPSS software (version 26.0), tabulated using Excel, and analyzed using Image-Pro Plus 6.0. $P < 0.01$ highly significant difference; $P < 0.05$, significant difference; and $P > 0.05$, no significant difference

RESULTS

RNA extraction results: RNA extracted from the longest dorsal and Leg muscles of Tibetan and Yorkshire Pigs was determined using an ultra-micro UV spectrophotometer. OD (optical density) values were between 1.8 and 2.2, which met the determination standard. The electrophoresis results are shown in Figure 1.

Differential expression of *FNDC5* in skeletal muscle of Tibetan and Yorkshire Pigs: To investigate the expression of *FNDC5* at the muscle level, 30, 90 and 180 days old Tibetan pigs and Yorkshire Pigs were selected, and the longest dorsal and Leg muscle tissues were selected for RT–qPCR experiments. In the longissimus dorsi muscle, the expression of *FNDC5* was significantly higher in 30, 90 and 180 days old Yorkshire Pigs than in Tibetan pigs.

Western blotting (Figure 3) revealed no significant difference between the pig breeds in longissimus dorsi

muscle and Leg muscle tissues at 30 days. Expression levels of *FNDC5* in the longissimus dorsi muscle and Leg muscle tissues of Tibetan pigs were significantly and highly significantly lower than those of Yorkshire Pigs at 90 and 180 days of age, both of which had a significantly higher expression level at 30 days of age than at 90 days of age. The expression level was significantly higher at 30 days than at 90 days of age, and the trend was the same in both tissues.

Skeletal muscle sections and HE staining: As shown in Figure 4, HE staining of both Tibetan and Yorkshire Pigs showed a variety of muscle fiber sizes and shapes; dark blue flat elliptical nuclei could be observed around muscle fibers. HE staining of skeletal muscle at 180 days of age showed that the diameter and area of muscle fibers of Tibetan pigs were significantly smaller than those of Yorkshire Pigs.

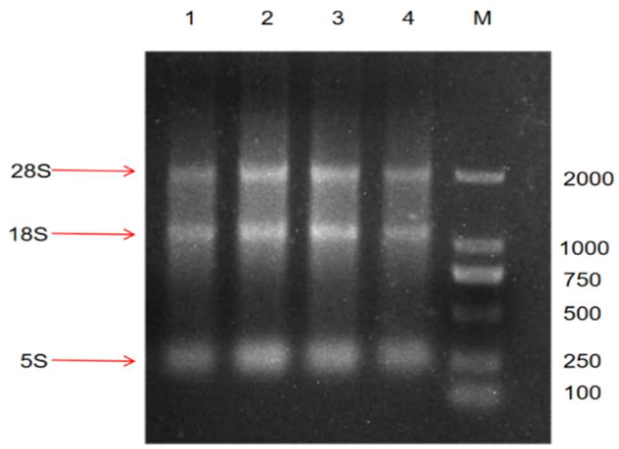

Figure 1. Results of total RNA extraction from tissues. M.D-2 000 markers 1–2 are Tibetan pigs 3–4 are Yorkshire Pigs.

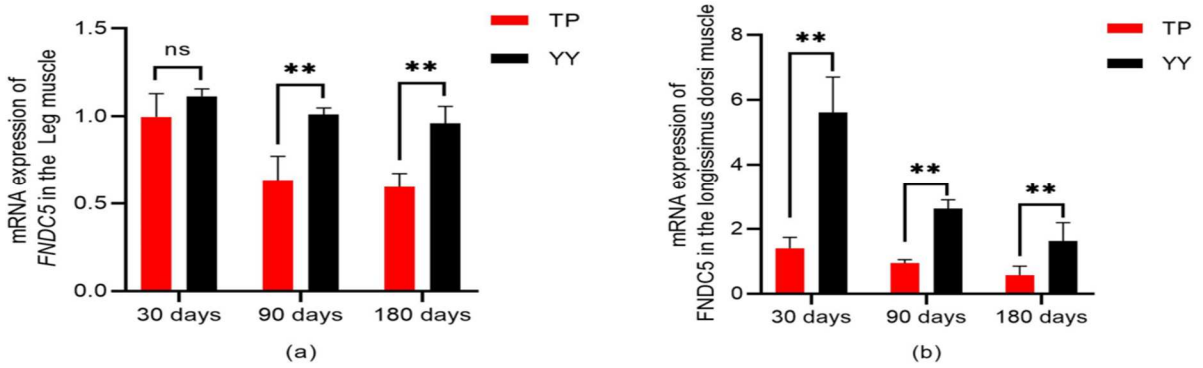

Figure 2. mRNA expression of *FNDC5* in the Leg and longissimus dorsi muscle of Tibetan and Yorkshire Pigs during different developmental periods. (a) Relative expression of *FNDC5* in Leg muscles at 30, 90 and 180 days of age. (b) Relative expression of *FNDC5* in the longissimus dorsi muscle at 30, 90 and 180 days of age. ns, no significant difference ($P > 0.05$); ** highly significant difference ($P < 0.01$)

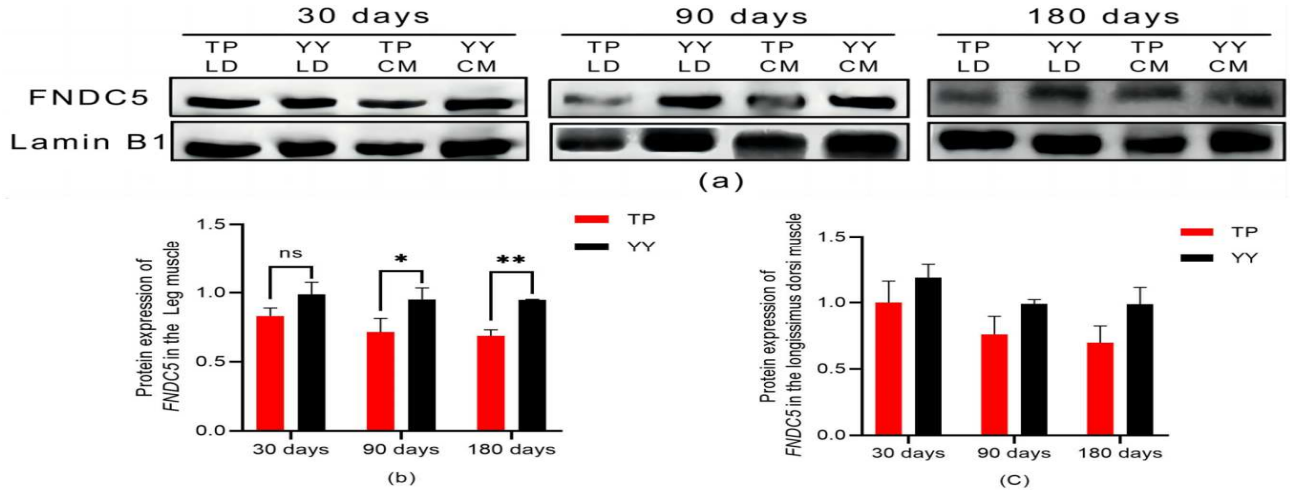

Figure 3. Protein expression of the *FNDC5* in Leg and longissimus dorsi muscles of Tibetan and Yorkshire Pigs during different developmental periods. (a) Protein expression of *FNDC5* in the Leg and longissimus dorsi muscles at different developmental periods by western blotting. (b) Protein expression of *FNDC5* in Leg muscle tissue. (c) Protein expression of *FNDC5* in longissimus dorsi muscle tissue. ns, no significant difference ($P > 0.05$), * significant difference ($P < 0.05$), ** highly significant difference ($P < 0.01$).

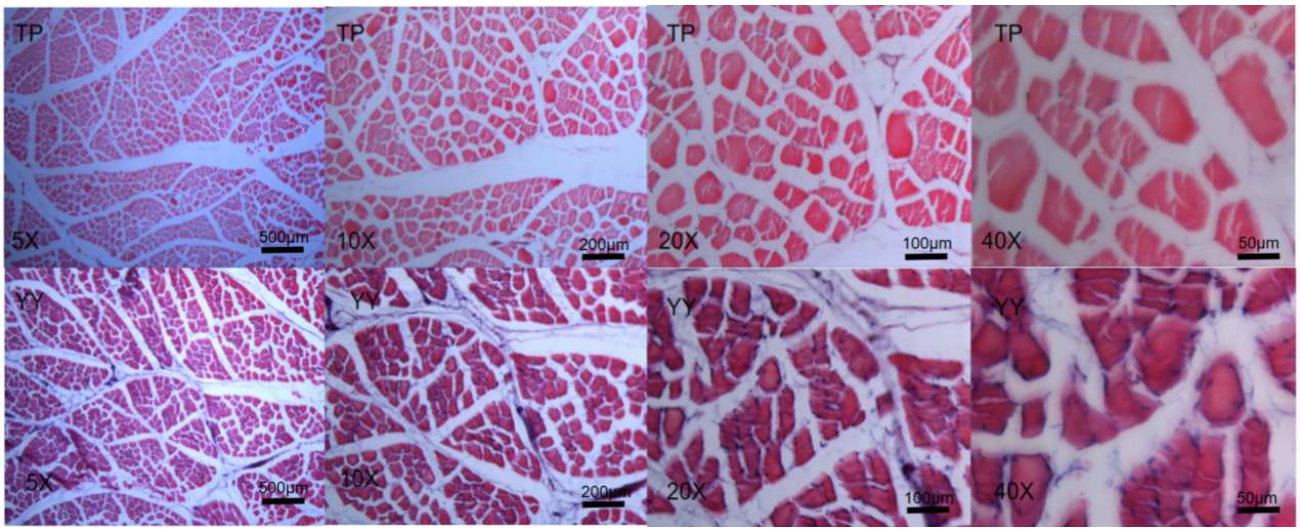

Figure 4. HE staining results of the longissimus dorsi muscle tissue of Tibetan and Yorkshire Pigs at 180 days of age (magnification 5/10/20/40 times). TP, Tibetan pig; YY, Yorkshire Pig.

Diameter and area of longissimus dorsi muscle fibers in Tibetan and Yorkshire Pigs: The longissimus dorsi muscle tissue was collected at 180 days of age, in which the muscle fiber diameter was 35.854 ± 1.564 μm in Tibetan pigs and 73.181 ± 2.611μm in Yorkshire Pigs (Table 2). The muscle fiber diameter of Tibetan pigs was significantly smaller than that of Yorkshire Pigs. The myofiber areas were 708.578 ± 9.085 ± m^2 in Tibetan pigs and 3297.038±190.696 ± m^2 in Yorkshire Pigs, and the difference was significant.

Table 2. Longissimus dorsi muscle fiber cross-sectional area and diameter in Tibetan and Yorkshire Pigs at 180 days of age.

Category	Tibetan pig (TP)	Yorkshire Pig (YP)	P-value
Muscle fiber diameter	35.854 ± 1.564 μm	73.181 ± 2.611 μm	0.000254
Muscle fiber area	708.578 ± 9.085 μm	3297.038 ± 190.696 μm	0.000171

DISCUSSION

FNDC5, a transmembrane protein found primarily in skeletal muscles, is a novel muscle growth factor released throughout the body as a product of the *FNDC5* gene (Boström *et al*. 2012). Irisin is regulated by peroxisome proliferator-activated receptor coactivator-1 (*PGC-1α*) and is thought to mediate the beneficial effects of exercise metabolism (Boström *et al*. 2012). Circulating irisin concentrations are negatively correlated with horizontal lipocalin and positively correlated with body mass index (BMI), fasting glucose, and total cholesterol levels, suggesting that the expression of *FNDC5* increases with an increase in muscle content in the body(Huh *et al*. 2012). In humans, muscle *FNDC5* expression increases with obesity(Timmons *et al*. 2012). The decrease in iris content after bariatric surgery does not directly lead to weight loss, probably because it increases energy expenditure and has a compensatory effect on insulin sensitivity(Ballantyne, Gumbs, and Modlin 2005; Vijgen *et al*. 2012). Estradiol and testosterone also induce an increase in muscle protein synthesis (Vijgen *et al*. 2012; Greising *et al*. 2011). In healthy females, estradiol levels are positively correlated with circulating irisin(Brown 2008). Estradiol can directly induce irisin secretion or act through anabolic pathways to increase muscle mass and upregulate irisin levels.

Muscle is an important factor in the maintenance of normal locomotion and physiological metabolism in pigs and is also an important indicator for assessing pig performance. Several genes regulate muscle growth and development.(Chen *et al*. 2021) Muscle fibers are the basic units of muscles, and their diameter, area, and number can significantly affect muscle quality.(Barbat-Artigas *et al*. 2013) The diameter, area, and number of muscle fibers can significantly affect muscle quality. The Tibetan pig is a typical small, lean pig breed found on a plateau;(Ma *et al*. 2019) Yorkshire Pigs are a large invasive pig breed with wide distribution worldwide.(Roth *et al*. 2022) *FNDC5* is abundantly expressed in muscle tissues and affects muscle metabolism by regulating lactate production, fat oxidation, and mitochondrial biosynthesis.(Lavi *et al*. 2022)

In this study, Tibetan and Yorkshire Pigs of different ages were selected as experimental animals to investigate the regulatory mechanisms and expression levels of *FNDC5* at the muscle level. First, RNA and proteins were extracted from the longest dorsal and Leg muscle tissues of the two pig breeds at 30, 90, and 180 days of age, and RT–qPCR and western blotting assays were used to detect their distribution. The results showed that in the Leg muscle tissues, the expression of mRNA and protein was highest in Tibetan pigs at 30 days of age, and although there was no significant difference between the two pig breeds, the trend was higher in Tibetan pigs; the expression of *FNDC5* was extremely significantly lower in Tibetan pigs than in Yorkshire Pigs in the RT–qPCR results at 90 days of age. Western blotting showed that mRNA and protein expression of *FNDC5* in Tibetan pigs at 180 days of age were both significantly lower than those in Yorkshire Pigs, and the lowest level was found at 30 days of age. In the longissimus dorsi muscle tissue, mRNA expression of *FNDC5* was significantly higher in the 30 days Yorkshire Pigs than in the Tibetan pigs; the protein expression was not significantly different, although the trend was the same as that of the quantitative results. mRNA expression of *FNDC5* was significantly higher in the 90, and 180 days of age Tibetan pigs than in the Yorkshire Pigs, and the protein expression was significantly higher in the Tibetan pigs. The protein expression was significantly higher in Yorkshire Pigs than in Tibetan pigs. In both muscle tissues, the expression of *FNDC5* was found to be most significant at a young age; expression decreased gradually with increasing age at the later stage, which was consistent with the results of RT–qPCR and western blotting.

The results of this study indicate that *FNDC5* exhibits a negative regulatory effect on muscle fiber traits in pigs; Gal(Lavi *et al*. 2022) *et al*. showed that the expression level of muscle *FNDC5* depends on fiber type and activity type, further validating the results of this study. It has been shown that the expression of the *FNDC5* in muscle is positively correlated with body mass but negatively correlated with age, and that age can be independently involved in regulating the expression of *FNDC5* in muscle after controlling for two variables: body index and sex (Nie *et al*. 2020). This phenomenon may be related to the growth and developmental demands of juvenile life and changes in the physiological status of adulthood, when animals generally have higher metabolic rates and require the largest amounts of energy and nutrients for growth and development(Speakman 2005) *FNDC5* is involved in muscle and energy metabolism(Boström *et al*. 2012) and its high expression during the juvenile period meets the energy and nutrient requirements of the body. As the body's metabolic rate decreases with age, the need to maintain muscle mass and energy reserves also decreases; therefore, the expression level of *FNDC5* may decrease. However, this needs to be confirmed through further experiments and studies.

Skeletal muscle sections and HE staining showed that the diameter and area of muscle fibers in Tibetan pigs were significantly smaller than those in Yorkshire Pigs, and the area of muscle fibers in Tibetan pigs was significantly lower than that in Yorkshire Pigs. Dinas *et al*.(Dinas *et al*. 2017) showed that the expression of *FNDC5* is significantly reduced in the muscle tissue of obese patients, resulting in a decrease in muscle-fat regulation. In this study, we also analyzed muscle fiber types in muscle tissues of Tibetan and Yorkshire Pigs in combination with phenotype-skeletal muscle sections and found that the muscle fiber diameter and area of Tibetan pigs were significantly smaller than those of Yorkshire Pigs. The body size of animals is directly proportional to muscle fiber diameter and area, which means that the higher the expression of *FNDC5*, the larger the size of animals and the higher the meat yield.(Prasad and Millay 2021) A small diameter of muscle fiber, large numbers of fat cells, and wide area of distribution are the main reasons for high muscle tethering power, juiciness, and flavor of meat.(Lawrie 1970) This is consistent with the apparent trait that Tibetan pigs are more tender, juicy, and flavorful than Yorkshire Pigs.(Cheng *et al*. 2015). In summary, *FNDC5* negatively regulates muscle fiber traits in pigs, improving pork production efficiency, pork quality, and sustainable development of animal husbandry, By using FNDC5 to manage the livestock, we can shorten the livestock cycle and increase production, thereby enhancing the economic efficiency of meat products. Traditionally, antibiotics have been vital to improving productivity and preventing disease in the livestock industry. However, these practices can pose serious health and environmental risks(Hu and Cheng 2016). By managing FNDC5, we can boost the immunity and health of livestock, thereby reducing the dependence on antibiotics.

Conclusion: In this study, we used RT–qPCR and western blotting to analyze the expression level and patterns of *FNDC5*, and found that the longissimus dorsi muscle and Leg muscle tissues are significantly different between Tibetan and Yorkshire Pig breeds at different ages. *FNDC5* expression is highest at 30 days of age, and then decreases gradually with growth and development. Differences in muscle fiber diameter, area, and density between the two species, analyzed by paraffin sectioning and HE staining of the longissimus dorsi muscle tissue of pigs at 180 days of age, suggests that *FNDC5* might be related to muscle growth. These results provide a reference for future research on meat quality enhancement in pig breeds on the Qinghai–Tibet Plateau.

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