

# Expression Analysis of Irisin During Different Differentiation Stages of Skeletal Muscle Cells

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## Research Article

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**Title page**

**Expression analysis of irisin during different differentiation stages of  
skeletal muscle cells**

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## **Abstract**

**Background:** As a newly discovered muscle factor secreted by skeletal muscle cells, irisin is a polypeptide fragment formed after hydrolysis by fibronectin type III domain-containing protein 5 (FNDC5). Previous studies have shown that irisin has biological functions that promote beiging of WAT, regulate glucose and lipid metabolism. However, the functions of irisin in muscle development and muscle fat metabolism remains unknown. **Results:** In order to study the expression of irisin in different growth stages of skeletal muscle, this study used SPF mice as experimental subjects to select skeletal muscle cells and muscle tissues of different developmental stages of mice. The expression of irisin precursor FNDC5 in different stages of cells and tissues was detected by western blotting and real-time fluorescent quantitative PCR, and the expression of FNDC5 in cells was detected by immunofluorescence. The results showed that FNDC5 was expressed in all stages of tissues and cells, but the expression was different at different stages. FNDC5 protein has the highest expression in muscle of sexually mature mice, followed by elderly mice and adolescent mice, and low expression in pups. Secondly, FNDC5 protein is mainly expressed in cytoplasm and highest in muscle fibers. The myotubes were the second, and the lowest in C2C12 cells. **Conclusions:** This experiment can provide a theoretical basis for the subsequent study of irisin in skeletal muscle, and lay the foundation for targeted therapy of related diseases.

## **Background**

As the main body of energy consumption, skeletal muscle accounts for 40% of the mammal's total weight. It is a highly adaptable tissue and plays a key role in the body's glucose and lipid metabolism. Irisin is a regulatory actin that is mainly secreted by skeletal muscle. It not only plays a local role in the muscle, but also acts on the systemic organs such as fat, liver, muscle, bone and brain in the form of paracrine or autocrine(1). Irisin produced by skeletal muscle plays a vital role in the development of insulin resistance and type 2 diabetes. Spiegelman first discovered irisin as the muscle factor that promotes the conversion of adipose tissue from white adipose tissue (WAT) to brown adipose tissue (BAT), which is a polypeptide of about 110 amino acids formed by the N-terminal hydrolysis of the FNDC5 protein(2), the upstream element of this fragment is PGC-1 $\alpha$ , which is an essential factor for energy regulation; the downstream is PPAR $\alpha$ , which activates the UCP1 gene and accelerates the body metabolism and energy expenditure, mutual union of upstream and downstream regulates the expression of irisin(3). The synthesis and secretion of irisin in skeletal muscle is affected by exercise, hunger, cold stimulation, and other factors. Among these, exercise is an important factor. Studies have shown that exercise induces increased expression of FNDC5, which increases the irisin shear released by FNDC5. Irisin is released through the blood circulation and reaches the energy-consuming tissues and organs such as the heart, brain, liver, and fat to act(4). Previous studies have shown that irisin has biological functions that promote beiging of WAT, regulate glucose and lipid metabolism, and improve insulin resistance. Later investigations have found that irisin also plays an important role in metabolic diseases of the liver, brain, and bones(5). Studies have shown that irisin can improve insulin resistance and maintain metabolic balance by increasing glucose uptake and oxidative utilization of fatty acids by skeletal muscle cells(6). In addition, irisin can also be used as a muscle-derived energy signal, increase the number and density of mitochondria,

accelerate the rate of oxidative metabolism in the body, promote glucose metabolism and energy expenditure, and play a role in coordinating skeletal muscle metabolism and maintaining the body's homeostasis(7, 8). Therefore, the increase in irisin is conducive to increasing of energy expenditure, it can inhibit the occurrence of obesity and insulin resistance, is a new target for the treatment of metabolic diseases, and has important research significance.

Irisin regulates the activity of muscle cells in an autocrine or paracrine manner(9). Mice injected with irisin can promote the synthesis of FNDC5 by myofibroblasts. In vitro, irisin stimulated C2C12 fibrovascular cells and significantly increased PGC-1 $\alpha$ , NRF1 and TFAM expressions were detected, leading to increased mitochondrial content and oxygen consumption. To date, studies have shown that mice injected with recombinant irisin have more FNDC5<sup>+</sup> fibers than mice injected with vector(10), suggesting that the secretion of irisin is promoted by enhancing the expression of irisin lysate precursors. In addition, some studies treated the C2C12 myotubule with 3.1-12.4 ng/mL irisin for 24 h, and observed that the expression of specific mitochondrial transcription factors increased, mitochondrial density and oxygen consumption increased(11). Studies have also shown that FNDC5 mRNA and irisin synthesis increase during myoblast differentiation of human myocytes in vitro, supporting the myoblast potential of irisin(1). In addition, IGF-1 levels were increased and myostatin levels were decreased in human myocytes treated with recombinant Irisin, whose expression was regulated by Irisin through ERK-dependent pathways(12). Reza MM et al. showed that exogenous injection of recombinant irisin could promote muscle hypertrophy and enhance muscle grip strength in MDX mice. Irisin treatment also reduced fibrosis and necrosis in dystrophic muscle fibers. In addition, Irisin improved muscle fiber membrane integrity in MDX mice without dystrophin. These demonstrate the therapeutic benefits of Irisin in improving muscle mass, muscle healing, and muscle function in a malnourished mouse model(13). Studies have shown that injection of Irisin in mice induces significant hypertrophy and enhances grip strength of uninjured muscles, and improves muscle regeneration and induces hypertrophy after skeletal muscle injury. The effect of Irisin on skeletal muscle hypertrophy is due to the activation of satellite cells and the enhancement of protein synthesis. In addition, Irisin injection rescues the loss of skeletal muscle mass after denervation by enhancing satellite cell activation and reducing protein degradation. These data suggest that Irisin acts as a myogenic factor in mice(1).

Obesity caused by excess energy is one of the main causes of various diseases such as heart disease, hypertension, diabetes, etc. Reducing food intake and increasing energy consumption to prevent excess calories from being converted to fat is effective method to reduce such problems. Studies have shown that irisin not only plays an important role in preventing obesity, reducing hyperglycaemia, improving diabetes and insulin resistance(12-15), but also plays a key role in metabolic regulation such as inflammation, hippocampal neurogenesis, and senescence(6). After the stimulation of exercise, cold and hunger induced the hydrolysis of FNDC5 to produce irisin, it can promote the browning of WAT and drive the generation of heat, which was once considered as the messenger between exercise and generation of heat(16). However, the receptor of irisin and the mechanism and signal transduction pathway of phenotypic transformation of WAT cells to BAT cells are still unclear. Some researchers believe that irisin promotes

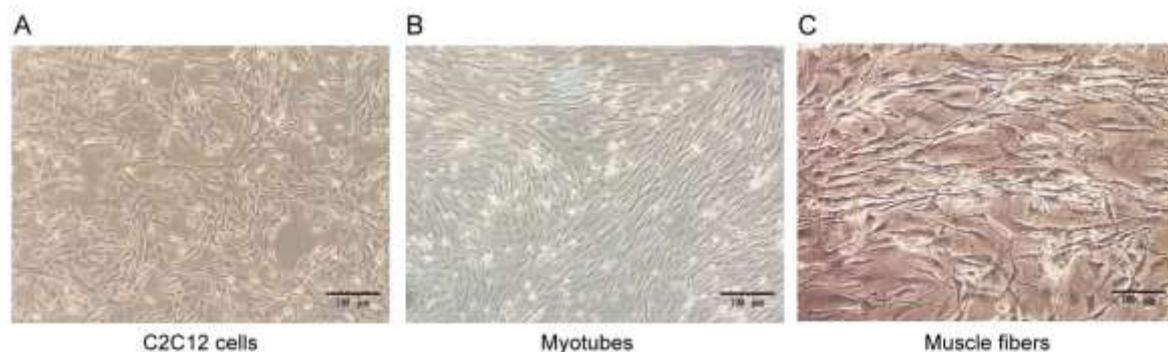
besieging of adipocytes by activating the P38/ERK MAPK signalling pathway and other signalling pathways by binding to unknown receptors on adipocytes to increase mitochondrial density and UCP1 expression *in vivo*(17). The expression of irisin *in vivo* is beneficial to reduce obesity and hyperglycaemia, improve diabetes and insulin resistance(18). Therefore, we intend to study the expression of irisin in tissues and differentiated cells at different stages of development by detect FNDC5, explore its expression characteristics *in vivo*, and provide new ideas for the treatment of fat metabolism diseases and complications.

By studying the expression of FNDC5 in skeletal muscle tissues and cells of mice at different stages of development, this study explored the expression pattern of irisin in skeletal muscle tissues and cells of mice, aiming to reveal the expression mechanism of irisin during the growth and development of skeletal muscle, and provide theoretical basis for irisin to improve the symptoms of metabolic diseases.

## Results

### Observation on the differentiation morphology of skeletal muscle

The attached C2C12 cells were spindle-shaped with irregular arrangement, as shown in Fig.1A. After 5 days of differentiation, the cell arrangement begins to show directionality, and multiple nuclei are in the centre, forming myotubes, like Fig.1B. After primary culture of muscle fibers, thin and long fibrous cells can be seen, as shown in Fig.1C.

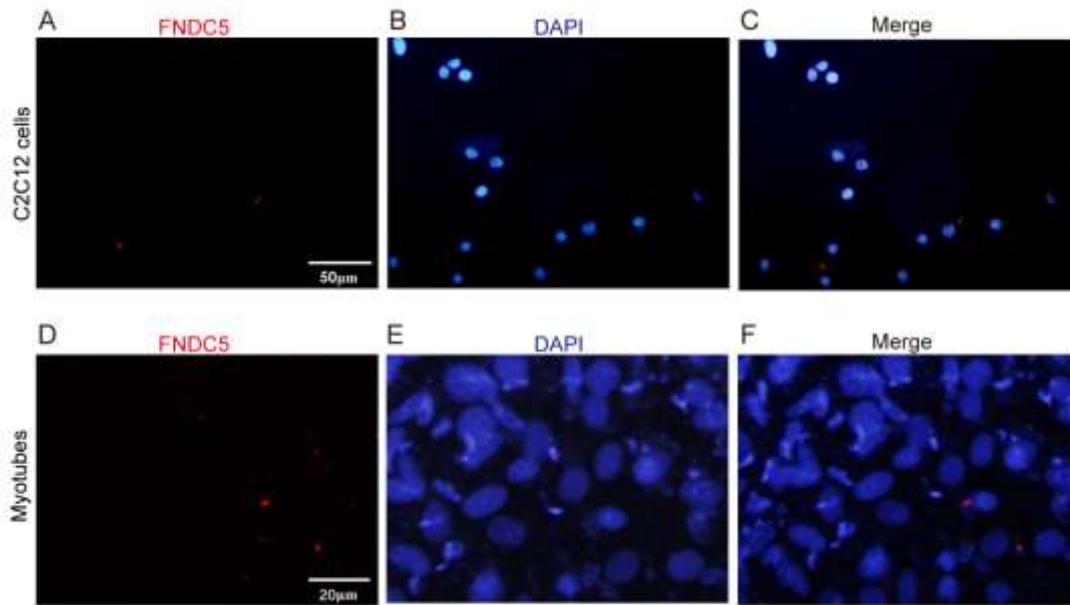


**Fig.1 Morphological changes of skeletal muscle cells at different stages of differentiation**

(A) Myoblast line C2C12 cells. (B) Differentiated myotubes. (D) Primary cultured muscle fibers.

### Localization of FNDC5 in cell

Immunofluorescence staining was performed on C2C12 cells and myotubes. Microscopic observation showed that the red light emitted by FNDC5 protein did not coincide with the blue light of the nucleus as shown in Fig.2, indicating that FNDC5 was expressed in the cytoplasm.

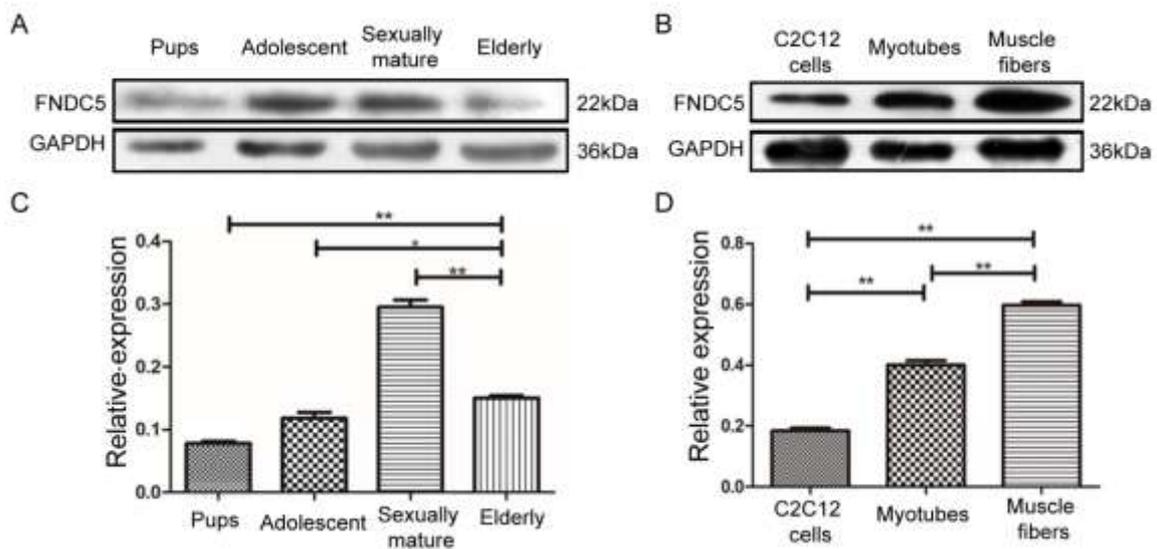


**Fig.2 Immunofluorescence localization of FNDC5**

(A-F). Immunofluorescence was performed on the C2C12 cells and myotubes. Immunofluorescence of FNDC5 was performed. DAPI (blue) shows nuclei. Scale bar represents 50  $\mu\text{m}$  or 20  $\mu\text{m}$ .

**Expression of FNDC5 protein in skeletal muscle cells and tissues of mice at different growth stages**

Western blot results of skeletal muscle tissues and cells were analysed, as shown in Fig.3. In the tissues, FNDC5 expression was the lowest in the pups and increased slightly at adolescence, and was significantly higher at sexual maturity than at other stages. In the elderly, FNDC5 expression was lower than at sexual maturity ( $P < 0.01$ ) and slightly higher than at adolescence ( $P < 0.05$ ) (Fig.3A&C). As shown in Fig.3B&D, the expression level of FNDC5 in muscle fibers was significantly higher than that in other stages ( $P < 0.01$ ), and that in myotubes was significantly higher than that in C2C12 cells ( $P < 0.01$ ).

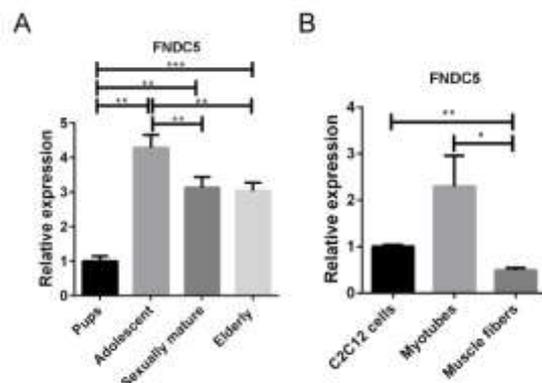


### Fig.3 Relative expression levels of FNDC5 protein in skeletal muscle cells and tissues at different growth stages in mice

(A&B) FNDC5 protein levels were measured by western blotting in skeletal muscle cells and tissues at different growth stages in mice. GAPDH was used as the control for the examination of FNDC5. (C&D) Semi-Quantitative Analysis of FNDC5 protein. Among skeletal muscle tissues at different developmental stages, FNDC5 is highest expressed during sexual maturity and lowest in pups ( $n = 3$ ,  $**P < 0.01$ ). Among skeletal muscle cells at different stages of differentiation, FNDC5 expression was highest in muscle fibers, and C2C12 cells had the lowest expression ( $n = 3$ ,  $**P < 0.01$ ). Data are expressed as means  $\pm$  SD,  $n=3$  per group containing three replicates.

### Expression of FNDC5 mRNA in skeletal muscle cells and tissues at different growth stages of mice

According to the qRT-PCR results, the experimental data were processed with statistical methods, and the results showed that FNDC5 was expressed in skeletal muscle at different developmental stages of mice, and the expression was different. Expression of FNDC5 in adolescent, sexually mature and elderly mice was significantly higher than that in pups, while its expression in adolescent mice was significantly higher than that in sexually mature and elderly mice (Fig.4A) ( $P < 0.01$ ). FNDC5 was expressed in three different differentiation stages of skeletal muscle cells (Fig.4B), its expression in myotubes was significantly higher than that in C2C12 cells and muscle fibers ( $P < 0.05$ ), while its expression in muscle fibers was significantly lower than that in C2C12 cells ( $P < 0.01$ ).



### Fig.4 Relative expression levels of FNDC5 mRNA in skeletal muscle cells and tissues at different growth stages in mice

(A) qRT-PCR analysis was conducted to detect FNDC5 expression in skeletal muscle tissues at different developmental stages. ( $n = 3$ ,  $**P < 0.01$ ). (B) qRT-PCR analysis was conducted to detect FNDC5 expression in skeletal muscle cells at different stages of differentiation ( $n = 3$ ,  $**P < 0.01$ ). Data are expressed as means  $\pm$  SD,  $n = 3$  per group containing three replicates.

### Discussion

Irisin is a muscle factor secreted by muscle cells and was once considered as an adipokine due to its effect on fat(19). The secretion of irisin in skeletal muscles is affected by exercise factors. Exercise can not only improve the expression of the target gene PGC-1 $\alpha$  of irisin, but also regulate the activity of irisin through multiple signalling pathways(20, 21). Meanwhile, as a molecular messenger between skeletal muscle and

surrounding tissues and organs, irisin can promote the decoupling of oxidative phosphorylation during energy generation by inducing upregulation of UCP1, and release energy in a non-shivering form to regulate body homeostasis(22). So irisin is also thought as a bridge between exercise and metabolic homeostasis.

Skeletal muscle is one of the largest organs of the human body, accounting for 40% of the total volume(23). In 2017, Reza et al. found that irisin in skeletal muscle can increase myogenic differentiation and fusion of C2C12 cells by activating IL-6 signalling. After irisin treatment, the number of C2C12 cells increased significantly at 24 h, 48 h and 72 h, in order to further study the effect of irisin on myogenesis. Reza et al. treat the differentiated C2C12 cells with irisin, compared with the control group, exogenous irisin improved the differentiation of C2C12 cells. After 72 h and 96 h of differentiation, the number of myotubes and myotube fusion index were significantly enhanced(24, 25). This is consistent with the results of our cell test that the expression of irisin in myotubes is higher than that of C2C12 cells through mRNA and protein analysis of irisin precursor FNDC5. However, although the transcription level of FNDC5 in muscle fiber was significantly decreased, its protein expression was still significant. Therefore, we speculate that irisin may promote the differentiation of muscle cells by stimulating related genes of muscle growth when the body is in the stage of muscle differentiation.

Martinez et al. used Northern Blot to respectively determine the total RNA in embryos and skeletal muscle cells of mice at 7 d, 11 d, 15 d and 17 d, it was found that FNDC5 mRNA level significantly increased after 15 days, and the expression of FNDC5 mRNA in myotubes was about 2 times higher than that in C2C12 cells(26). This suggests that FNDC5 expression is associated with different growth stages of the body, and FNDC5 expression is significantly up-regulated as the body develops and matures. According to our results, the expression level of FNDC5 was the highest in sexually mature mice, followed by elderly mice and adolescent mice, and the lowest in pups mice. This may indicate that the expression of irisin is significantly increased with the development and maturation of mice. The expression of irisin in skeletal muscles of elderly mice is significantly lower than sexually mature mice, which may be due to the decreased expression of irisin in the body caused by muscular atrophy in elderly mice. In conclusion, the expression level of irisin is closely related to the growth stage of skeletal muscle, and irisin may be a myotrophic factor.

Irisin is a regulator that is helpful in metabolism during exercise and plays an important role in the metabolic process of our body(27). According to the analysis of DNA, mRNA and gene sequence of FNDC5, FNDC5 is highly conserved in most primates, while the starting codon of FNDC5 gene in human is changed from ATG to ATA(28). Therefore, further work is needed to confirm our study of irisin in skeletal muscle of mice and its potential as a target for the treatment of metabolic diseases in humans.

## **Conclusion**

In brief, FNDC5 was expressed in skeletal muscle cells and tissues at different developmental stages of mice, and there were differences. The expression level was highest in the mature period of mice, but lowest in newborn mice. At the same time, FNDC5 was mainly expressed in cytoplasm, and the expression level was the highest in muscle fiber, followed by myotubes, and the lowest in C2C12 cells.

## **Methods**

### **Sample Collection**

This study was approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University, taigu, China and all procedures involving animal treatment and sample collection were performed by veterinarians following the Guiding Principles for animal use described by the Council for International Organizations of Medical Sciences (CIOMS). C57BL/6J male mice, pup's mice (7 days), adolescent mice (21-28 days), sexually mature mice (42-56 days) and elderly mice (more than 8 months), were purchased from Shanxi Provincial People's Hospital (mice were divided in four groups, n = 3 per group). The individual mouse was considered the experimental unit within the studies. All mice were housed in a specific pathogen-free standard room under controlled temperature (19–23 °C) and humidity (50±20%) conditions and a 12-h light–dark cycle. After the ankle annulus was cut, skin was removed along the incision, gastrocnemius muscle was separated, and the muscle was quickly frozen in liquid nitrogen for quantitative real-time polymerase chain reaction (qRT-PCR) and Western Blot analysis.

### **Cell culture**

Cell culture: Proliferating C2C12 murine myoblasts (ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/L D-glucose, 4.0 mM L-glutamine, 1.0 mM sodium pyruvate; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) at 37 °C, in 95% air and 5% CO<sub>2</sub>. Myogenic differentiation was induced when the C2C12 cells density reached 50-60%, by replacing growth media with the differentiation medium [DMEM, 4.5 g/L D-glucose, 4.0 mM L-glutamine; Thermo Fisher Scientific, supplemented with 2% horse serum (HS; Thermo Fisher Scientific)]. Differentiation media was replaced every 24 h for the duration of differentiation.

Culture of primary muscle fibers: 8 newborn 14 days mice were sacrificed. The limbs of the mice were washed 4 times in a 1% penicillin mixture (Thermo Fisher Scientific), the skin, bones and connective tissue were removed, and 0.25% trypsin (Thermo Fisher Scientific) was added for digestion for 30 min. Collect the filtered cells in a centrifuge tube and maintain in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/L D-glucose, 4.0 mM L-glutamine, 1.0 mM sodium pyruvate; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) at 37 °C, in 95% air and 5% CO<sub>2</sub>. The C2C12 cell line was kindly gifted by China Agricultural University and the cell line was in the laboratory at the time of the described experiments about half a year. And we have confirmed that this cell cultures have been tested for mycoplasma and that the test was negative(29, 30).

### **RNA extraction and semi-quantitative real-time PCR**

Use TRIzol (TaKaRa Bio, Dalian, China) method to extract RNA from tissues and cells, cDNA was synthesized using the extracted RNA as a template according to the instructions of the reverse transcription kit (Vazyme, Nanjing, China). Specific primers were designed using Primer-BLAST (NCBI, NIH, Bethesda, MD, USA) according to the mouse FNDC5 and  $\beta$ -actin gene sequences published in GenBank (NCBI, NIH, Bethesda, MD, USA). All primers were synthesized by BGI (Shenzhen, China).

Real-time fluorescent quantitative PCR was performed using a 10  $\mu$ L system. The system was: 1  $\mu$ L of cDNA template, 3  $\mu$ L of DEPC water, 5  $\mu$ L of SYBR Premix Ex Taq (2 $\times$ ) (Takara Bio, Beijing, China), and 0.2  $\mu$ L of ROX Plus (Takara Bio, Beijing, China), 0.4  $\mu$ L each of the upstream and downstream primers. The upstream primer sequence of FNDC5 is TAGGCTGCGTCTGCTTCG, and the downstream primer sequence is TGTCTCCTCCAGGTC CCA. The  $\beta$ -actin upstream primer sequence is TTGTCATCACTAACGAGCGCA, and the downstream primer sequence is AACAGTCCGCCTAGAAGCAC. Each sample was tested in 4 independent replicates. The reaction process was: pre-denaturation at 95  $^{\circ}$ C for 10 min, denaturation at 95  $^{\circ}$ C for 30 s, annealing at 60  $^{\circ}$ C for 30 s, and extension at 72  $^{\circ}$ C for 20 s; a total of 40 cycles. The  $2^{-\Delta\Delta C_t}$  method was used to analyze the relative expression of FNDC5 gene at different growth stages of skeletal muscle.

### **SDS-PAGE and Western immunoblotting**

The total protein of the tissues and cells was extracted by Lysis Solution Kit (Beyotime, Shanghai, China). Total protein concentration was determined using a BCA protein assay (Bio-Rad Laboratories). The total protein loading amount of each sample was 200  $\mu$ g. After SDS-PAGE electrophoresis, Proteins were transferred onto nitrocellulose filter (NC) membranes by semi-dry transfer (Trans-blot Turbo, Bio-Rad Laboratories) at 1 amp for 10 min. Incubate with FNDC5 polyclonal antibody (Cat No. 23995-1-AP, Proteintech, Wuhan, China) and GAPDH Monoclonal antibody (Cat No. 60004-1-1g, Proteintech, Wuhan, China) at 4  $^{\circ}$ C overnight, then incubate with HRP-conjugated secondary antibody diluted in TBST at 37  $^{\circ}$ C for 1 h. Immunodetection was performed using ECL substrate (Thermo Fisher Scientific) on a ChemiDoc System (Bio-Rad Laboratories). Protein bands were analyzed using ImageLab software (Bio-Rad Laboratories), Analysis of the target protein were using ImageLab software (Bio-Rad Laboratories), Protein content = band area  $\times$  average gray; semi-quantitative value of target protein = target protein content/GAPDH protein content.

### **Immunofluorescence**

C2C12 cells and myotubes were grown on 18 mm round glass coverslips in 6-well cell culture plates. When the density of C2C12 cells and myotubes reached 60-70%, fixed with 4% tissue cell fixative (Solarbio, Beijing, China) for 30 min at room temperature, washed with PBS (3 $\times$ 5 min). Permeabilized with 0.1% Triton X-100 (Solarbio, Beijing, China) for 20 min at room temperature, then washed with PBS (3 $\times$ 5 min). Added goat serum (Solarbio, Beijing, China) to block in a 37  $^{\circ}$ C incubator for 30 min. 2% FNDC5 primary antibody (Cat No. 23995-1-AP, Proteintech, Wuhan, China) was used to incubate at 4  $^{\circ}$ C overnight. After rewarming at 37  $^{\circ}$ C for 45 min, washing with PBS (3 $\times$ 5 min). Add 0.5% fluorescent secondary antibody (Cat No. SA00013-4, Proteintech, Wuhan, China), incubate at 37  $^{\circ}$ C for 1 h, and wash with PBS (3 $\times$ 10 min) (protected from light). Microscopy after mounting with DAPI-containing anti-fluorescent attenuator (Solarbio, Beijing, China).

### **Statistical analysis**

Statistical analysis was performed using SPSS 19.0 for ANOVA. Data were presented as the means  $\pm$  SD, and the level of statistical significance was set at \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. All the columns were processed with GraphPad Prism<sup>TM</sup>.

## **List of abbreviations**

FNDC5, fibronectin type III domain-containing protein 5; WAT, white adipose tissue; BAT, brown adipose tissue.

## **Figure Legends**

### **Fig.1 Morphological changes of skeletal muscle cells at different stages of differentiation**

(B) Myoblast line C2C12 cells. (B) Differentiated myotubes. (D) Primary cultured muscle fibers.

### **Fig.2 Immunofluorescence localization of FNDC5**

(B-F). Immunofluorescence was performed on the C2C12 cells and myotubes. Immunofluorescence of FNDC5 was performed. DAPI (blue) shows nuclei. Scale bar represents 50  $\mu\text{m}$  or 20  $\mu\text{m}$ .

### **Fig.3 Relative expression levels of FNDC5 protein in skeletal muscle cells and tissues at different growth stages in mice**

(A&B) FNDC5 protein levels were measured by western blotting in skeletal muscle cells and tissues at different growth stages in mice. GAPDH was used as the control for the examination of FNDC5. (C&D) Semi-Quantitative Analysis of FNDC5 protein. Among skeletal muscle tissues at different developmental stages, FNDC5 is highest expressed during sexual maturity and lowest in pups ( $n = 3$ ,  $**P < 0.01$ ). Among skeletal muscle cells at different stages of differentiation, FNDC5 expression was highest in muscle fibers, and C2C12 cells had the lowest expression ( $n = 3$ ,  $**P < 0.01$ ). Data are expressed as means  $\pm$  SD,  $n=3$  per group containing three replicates.

### **Fig.4 Relative expression levels of FNDC5 mRNA in skeletal muscle cells and tissues at different growth stages in mice**

(A) qRT-PCR analysis was conducted to detect FNDC5 expression in skeletal muscle tissues at different developmental stages. ( $n = 3$ ,  $**P < 0.01$ ). (B) qRT-PCR analysis was conducted to detect FNDC5 expression in skeletal muscle cells at different stages of differentiation ( $n = 3$ ,  $**P < 0.01$ ). Data are expressed as means  $\pm$  SD,  $n = 3$  per group containing three replicates.

## **Declarations**

### **Ethics approval and consent to participate**

This study was approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University, Taigu, China and all procedures involving animal treatment and sample collection were performed by veterinarians following the Guiding Principles for animal use described by the Council for International Organizations of Medical Sciences (CIOMS).

### **Consent for publication: Not applicable**

### **Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files].

### **Competing interests**

The authors declare that they have no competing interests

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#### **Authors' contributions**

YY: Data Curation, Methodology, Writing-Original draft preparation. DY: Methodology, Writing-Original draft preparation. PW: Software, Validation. YL: Methodology. YG: Date Curation. PM: Formal analysis. JY: Data Curation. PZ: Resources. ZZ: Methodology. XL: Writing Review and Editing. XY: Methodology. HW: Conceptualization, Funding acquisition, Project administration.

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**Statement: I confirmed that the study is reported in accordance with ARRIVE guidelines.**

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