Original Article
Fndc5/irisin is regulated by myogenesis stage, irisin, muscle type and training

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Abstract: Objectives: Irisin, a novel myokine that responds to exercise, was initially identified as a regulator of fat tissue metabolism. We aimed to investigate fibronectin type III domain-containing protein 5 (Fndc5)/irisin, auto/para-crine role in different muscle fibers, different activities, and muscle cell differentiation. Methods: Using in-vitro, ex-vivo, and in-vivo muscle models, Fndc5 was studied at the physiological and molecular levels. Results: Following training, C57BL/6 mice (n=10) were subject to fast and slow-twitch muscles dissection and molecular analysis. Isolated mice (C57BL/6, n=14) slow and fast-twitch muscles were subject to electrical aerobic and anaerobic pulses stimulation (EPS). L6 muscle cells differentiation was characterized by Fndc5 differentiation-depended expression pattern parallel with significant hypertrophy, Myogenin elevation, and overlapping Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (Pgc-1α) expression pattern. Exogenous irisin significantly altered Fndc5 expression; augmented at early differentiation (3-4-fold, P<0.05) and decreased (2-fold, P<0.05) at late differentiation. Training induced a significant elevation in Fndc5/irisin and Pgc-1α expression levels in all muscle types compared to the sedentary state, where soleus muscle (slow) Fndc5 expression levels were significantly higher compared to all other fast muscles (3-140-fold, P<0.001). Similarly, following EPS, Fndc5 expression levels were significantly augmented in the soleus slow muscle following both aerobic and anaerobic activity (3-3.5-fold, P<0.05) compared to extensor digitorum longus (fast) muscle. Conclusions: Muscle cell’s Fndc5 expression has a differentiation-depended pattern paralleling Pgc-1α expression and hypertrophy, irisin autocrinally and significantly regulate Fndc5 and Pgc-1α in a differentiation-depended manner. Muscle Fndc5 expression levels are dependent on fiber type and activity type.

Keywords: Irisin, FNDC5, muscle, myogenesis, Pgc-1α

Introduction

Skeletal muscle tissue is highly flexible and responsive to endogenous and exogenous cues [1]. The tissue plays a vital role in whole-body metabolism in health and disease through constant interactive communication through an array of cytokines named myokines and other hormone-like peptides. The muscle myokines act through autocrine, paracrine, and endocrine mechanisms on muscle tissue and other tissues, including; adipose, liver, pancreas, bone, heart, immune and brain cells [2]. The autocrine and paracrine effects of myokines are mostly related to muscle physiology regulation, such as muscle hypertrophy and myogenesis, and muscle metabolism [3]. For example, myokines affect muscle satellite cell proliferation and hypertrophy in an adaptive response to physical activity [4-6]. The endocrine effect of myokines is mostly related to the whole-body effect of exercise on various tissues and mediates the whole-body health benefits of exercise [7]. For example, several studies suggested that myokines participate in the regulation of systemic glucose homeostasis and insulin sensitivity [8], improve brain function [9, 10], and encourage bone cell differentiation [11]. Thus, myokines are involved in muscle hypertrophy and myogenesis, exercise metabolism, glucose uptake, and fatty acid oxidation in the skeletal muscle. Skeletal muscle accounts for ~80% of whole-body glucose disposal under insulin-stimulated conditions [12]. Consequently, by autocrine mechanisms, myokines improve endocrine pancreas insulin secretion and
Irisin regulates muscle differentiation and activity

Irisin - a myokine hormone, is encoded by FNDC5 gene located on chromosome 1, and it was first identified in 2012 [14]. The FNDC5 gene encodes a transmembrane protein, which upon activation, releases its outer part, Irisin, into the blood [15, 16]. Although it was identified that the FNDC5 gene is regulated by PGC-1α [14] and AMP-activated protein kinase (AMPK) [17, 18], to this date, Irisin's receptor, regulation of release, and signal transduction pathways involved in its target tissues are largely unknown [14].

Initially, the role of Irisin was found to act on adipocytes, catalyzing their transformation to metabolically active brown fat cells. Consequently, resulting in significant boosts in the body's total energy expenditure and elevated resistance to the effects of insulin-related obesity and diabetes [14]. Furthermore, mildly increased circulating Irisin levels were found to cause increased energy expenditure in mice even with no changes in movement or food intake [16]. Thus, the interactions between muscle/adipose axes through irisin have important implications for fat and lean deposition and the efficiency of the human body energy utilization in growth and development [19].

Initial studies on the autocrine role of Irisin on muscle metabolism and hypertrophy suggest Irisin enhances myoblast fusion and stimulates muscle growth-related genes, indicating Irisin contributes to muscle growth and hypertrophy in response to physical activity [6, 20]. Yet, the role of Irisin in physical activity is limited and contradicting. Exercise induces Irisin in both mice and humans [21]. After intensive exercise, Irisin circulating levels increase maximum heart rate (HR) in both trained and untrained humans [22]. Muscle Irisin levels (not circulating) were also found to significantly increase following high-intensity interval aerobic training (HIIT) compared to classic aerobic exercises [23]. However, in contrast, blood Irisin concentration following HIIT and continuous endurance training (CET) significantly decreased compared to pre-training values [24]. Additionally, another study found no significant difference in muscle and serum Irisin levels after aerobic or anaerobic exercise [25].

Taking into consideration the limited information regarding the novel endo/para and autocrine role of Irisin in muscle and the complexity of the muscle cell/tissue, we aimed to study Fndc5/Irisin, auto/para-crine role in different muscle fibers and activities and during muscle cells differentiation using 3 models: in vitro, ex-vivo and in vivo.

Methods

Cell culture

Rat L6 myoblast cell line (ATCC, VA, USA) were grown as a sub-confluent culture maintained in Dulbecco's Modified Eagle's Medium (DMEM-alpha, Biological Industries (BI), Israel), supplemented with 10% fetal bovine serum (FBS, BI, Israel) and 1% penicillin-streptomycin (BI, Israel). Cells were induced to differentiate using a differentiation medium containing DMEM-alpha (BI, Israel), supplemented with 2% FBS (BI, Israel) and 1% penicillin-streptomycin (BI, Israel). The cell lines were cultured in an atmosphere of 5% CO₂ at 37°C.

Muscle fiber thickness test

Cultured L6 cells (200,000 cells/ml) were seeded on 12 well plates, subjected to differentiation and photographed through days of differentiation, at time points: days 1-10. Photography was done using a light microscope (Olympus microscope IX81). Hypertrophy was evaluated by measuring the muscle fibers' thickness (µm) using ImageJ software version 1.8.0_172.

Experimental animals

All animal procedures were carried out at Ariel University Animal Facility with approval from the Ariel University Animal Ethics Committee (IL-168-12-18). Male mice (C57BL/6), 6-8 weeks old, were purchased from Envigo Inc, Jerusalem, Israel) and were maintained under standard 12 hr: 12 hr light: dark conditions with ad libitum access to food and water. Post training, animals were sacrificed by rapid neck dislocation and muscle isolation was done followed by molecular analysis.

In vivo training protocol-physical training in the running track

Male mice (C57BL/6, n=10), 6-7 weeks old upon arrival, underwent 6-week treadmill high-intensity continuous training (HICT) programs on a 5-lane treadmill designed for mice (Panlab...
Harvard Apparatus, USA), as previously described [26]. Briefly, before the start of the training program, the mice underwent three days of treadmill adaptation (10 min/day) and reached a pace of 8 cm/second. The training program included initial three weeks (5 days/week, 23 cm/sec) training program consisting of a gradual increase in physical activity (running) and volume (speed and duration), followed by a HICT program for another three weeks (5 days/week) (Scheme 1). Each training session consisted of a 5-minute warm-up at 8 cm/sec. For the first, second, and third weeks, the warm-up was followed by 10, 20, and 30 minutes of training at 23 cm/min, respectively. In the subsequent three weeks, the trained mice were subjected to an incremental training protocol, reaching 23 minutes of training at 34 cm/min in the last two weeks. Based on the baseline exhaustion speed performance test (45±2 cm/sec; n=10), this training speed corresponds to an exercise intensity of 75% of maximal speed. As previously described, the HICT running speed was based on exhaustion performance tests [26]. Mice were subject to pre and post-training performance tests, exhaustion speed and exercise tolerance performance tests, and the intensities were individually adjusted to each mouse.

**Exhaustion speed performance test:** Maximal running speed was assessed first by running the mice for 8 cm/sec and then increasing the speed by 2 cm/sec per minute until exhaustion. Exhaustion was defined by an inability/refusal to continue when encouraged with a bottle brush or a small puff of air. We defined HICT as 70-75% of exhaustion speed.

**Exercise tolerance performance test:** Exercise tolerance was determined by running each mouse individually to exhaustion at 30 cm/sec on a rodent-specific treadmill. Exhaustion was defined as above.

**Electrical pulses stimulation system**

SOL and EDL muscles were isolated from both legs (4 samples from each mice) in contraction Tyrode solution buffer containing (mM): 121 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.5 mM NaH$_2$PO$_4$, 0.4 mM MgCl$_2$, 24 mM NaHCO$_3$, 0.1 mM EDTA, 5.5 mM glucose, constantly gassed with 5% CO$_2$:95% O$_2$ to yield a final pH of 7.4 at 25°C. Isolated mouse muscle for contraction was set up as previously reported [26]. Briefly, silk threads were tied to the tendons, and the muscles were transferred to a stimulation chamber (World Precision Instruments). Muscle ends were tied to hooks; one hook is fixed and the other is adjustable to optimize muscle length. The chamber was filled with a Tyrode solution. Ten min after mounting, the optimal length for generation of maximal isometric tetanic force was set with 100 ms trains at 70 Hz. Muscles were then allowed to recover for 20 min before starting the experiment.

**Stimulation protocol**

After an equilibration period of 30 min, EDL and SOL muscles underwent stimulation protocol. Muscles were repeatedly contracted for 10 min, with 1 contraction every 2 sec. Each contraction was 100 msec in duration and at a frequency of 50 Hz. Paired EDL and SOL muscles were exposed to aerobic or anaerobic conditions during contraction. The anaerobic condition was achieved by the addition of sodium cyanide (3 mM). At the end of 10 min, muscles were immediately frozen and stored in liquid nitrogen. All reagents were from Sigma and Roche.
RNA isolation

Total RNA was isolated using TRI reagent (Trizol, Rhenium, Israel) according to the manufacturer's procedure. Briefly, cells were homogenized in TRI reagent, stored at room temperature (RT) for 5 min, after which 200 µl chloroform (Ornat, Israel) was added, and samples were shaken for 10 sec. The mixture was stored at RT for 12 min and centrifuged at 12,000 RCF for 15 min at 4°C. Following centrifugation, the upper aqueous phase was precipitated by 500 µl Isopropanol (Ornat, Israel), incubated for 10 min at RT, and centrifuged at 12,000 RCF for 10 min at 4°C. The palates were precipitated in 75% ice-cold ethanol and centrifuged at 7,500 RCF for 5 min at 4°C. After which, samples were air dried for 10 min and dissolved in ultra-pure water for 15 min at 60°C. RNA was quantified by UV absorption using a spectrophotometer (UV-Vis spectrophotometer; NanoDrop 2000c Thermo Scientific, USA). Samples were stored at -80°C.

Reverse transcription (RT) PCR

Total RNA was converted to cDNA using Tetro enzyme (Lifegene, Israel) according to the manufacturer protocol with slight modifications. Briefly, 200 U reverse transcriptase was added to 1 µg of total RNA and 0.2 µg random hexamers (Lifegene, Israel) to a final volume of 20 µl containing 2 mM NTP mix (Lifegene, Israel) and 5X first-strand buffer (Lifegene, Israel). The RT reaction was performed at 42°C for one hr and inactivated by heating to 70°C for 10 min. Samples were stored at -20°C or were used immediately for qPCR.

Real time quantitative PCR (qPCR)

Transcripts levels were determined using qPCR (AriaMx G8830A Agliment instrument) using SYBR green PCR Master Mix (Rhenium, Israel) according to manufacturer's protocol. All primers were designed across exons to prevent false negative results. cDNA and primer concentrations were optimized (including melting curve analysis). Each 20 µl reaction contained; 2 µl cDNA, 10 µl PCR master mix, forward and reverse primers, and ultra-pure water. qPCR reactions were performed under the following conditions: pre-incubation at 50°C for 10 min, denaturation at 95°C for 10 min, and 40 cycles of 95°C followed by annealing and elongation at 60°C for 1 min. Gapdh, Actin, and S18 (housekeeping genes) were used for mRNA level normalization.

Primer design for qPCR

The mRNA sequence of each target gene was taken from NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/). Gene specific primers were designed using the Primer online program (http://bioinfo.ut.ee/primer3-0.4.0/) and purchased from Sigma-Aldrich (Jerusalem, Israel). The primers used for qPCR were listed in Table 1.

Protein isolation

Proteins were extracted using RIPA lysis method. In brief, cells were suspended in 50 µl RIPA lysis buffer (15 mM Tris-HCl, 1% Triton X100, 0.1% SDS, 167 mM NaCl, 0.5% Sodium Deoxycolatic acid), containing 2% protease inhibitor cocktail (Sigma Aldrich, Israel). After samples were incubated for 30 min on ice and centrifuged at 14,000 RPM for 20 min at 4°C. Protein quantification was measured by Bradford assay [27]. Samples were stored at -80°C.

Western blot

Equal concentrations of protein samples were prepared with 5X loading buffer for final volume of 30 µl. Samples were heated at 95°C for 5 min and separated on 10-16% SDS gel page followed by transference to nitrocellulose mem-

| Table 1. Primers used for qPCR |
| Mouse Gapdh | F-GTCTCCCTGTGACCTCAACAGG | R-ACCACCCTCTTGCAAGGGCA |
| Mouse Actin | F-GCACCCACCTTCTTCAATG | R-TGCTTGCTGACCACACATCG |
| Mouse Fndc5 | F-ACAGGCAGAGAGCAGAGACG | R-GAACTGTGCTGACACATCGA |
| Mouse Pgc-1α | F-GGTGCCCTCTCAGTCTCTC | R-ACAGAGACAGACACATCTT |
| Rat S18 | F-GTAACCGGGTGAACCCCCC | R-CCATCCTACGGTGTAAGCG |
| Rat Myogenine | F-TGCACATCTGTGCAGTCTCCC | R-CCTATATGCTCCCTCCTC |
| Rat Fndc5 | F-AGAAGTCCATGCAACACC | R-GGGAACCTGTGAGGAG |
| Rat Pgc-1α | F-CGCTTCCACCCGATCATT | R-CTCTGTCCTCCCTCACAGGAC |
brane. After washing with Tris buffer saline with Tween 20 (TBST), blocking was done using 3% dry milk solution for 1 hr. Membranes were incubated with primary antibody overnight at 4°C. After which, blots were washed once for 15 min and twice for 5 min with TBST. Secondary antibody was incubated for 1 hr, followed by 4 times, 5 min wash with TBST. Detection was done using 2 min incubation with E-Z-ECL chemiluminescence detection kit (EZ-ECL, BI, Israel). After exposure to ECL, bands were detected using ImageQuant LAS4000 Mini (GE Healthcare, Life Science, Israel). The specific bands were subjected to densitometry analysis ImageJ software (1.46 version). All proteins were quantified relatively to housekeeping Actin protein.

**Antibodies list**

Mouse Anti β-Actin 1:5000, (MBA1501; Mercury, Germany); Rabbit Anti FNDC5 1:3000, (ab174833; Abcam, USA); Rabbit Anti PGC-1α 1:1000, (ab54481; Abcam, USA); Goat Anti Mouse IgG HRP Conjugate 1:3000, (ab6789; Abcam, USA); Goat Anti Rabbit IgG HRP Conjugat 1:3000, (ab6721; Abcam, USA).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (version 7.04). Data are expressed as mean ± SD. Results were expressed as mean ± SE. Results were considered significantly different if P-value (P) were less than 0.05 (α=0.05). Comparisons between gene and protein expression levels during differentiation were analyzed using one-way ANOVA, followed by Tukey’s post hoc test. Inter-group differences comparing the effects of irisin treatment and trained vs. untrained mice were analyzed using two-way ANOVA, followed by Tukey’s post hoc test.

**Results**

**Fndc5 and Pgc-1α genes expression during differentiation of L6 muscle cells in-vitro model**

We initially analyzed skeletal muscle morphology during differentiation using in vitro L6 skeletal muscle model system [28, 29].

The morphology of undifferentiated pre-differentiation induction is demonstrated on days-2-0, where cells exhibit round shapes (Figure 1A). As differentiation was induced (day 1), cell morphology alterations were noted (Figure 1A). We found a significant increase in the mean thickness of the muscle fibers, with 10% average increments from day to day during L6 differentiation, reaching maximum significant mean muscle fiber thickness at day 8 of differentiation. Altogether, the mean thickness of the muscle fibers was significantly elevated by 200% from day one to day 8-10 of differentiation (Figure 1B). In parallel, maximal muscle fiber thickness increased significantly through L6 differentiation days, with an average of 10-30% increments between days, reaching a maximum peak at the day 8 of differentiation. The maximal thickness of the muscle fibers was significantly elevated by 300% from day one to day 8-10 of differentiation (Figure 1C). These results demonstrate that the L6 muscle cell model exhibits a classical phenotype of differentiated muscle cells. Accordingly, the expression of Myogenin, a well-established marker for muscle differentiation known to be associated with muscle hypertrophy, increased significantly during L6 cell differentiation by 170% and 230% on days 2 and 7, respectively, compared to levels at day 0 (Figure 1D).

As shown in Figure 1E, Fndc5 transcripts levels were significantly increased by 530% and 200% on days 5 and 7 of differentiation, respectively, compared to day 0, reached significant peak levels at day 5 of differentiation. Corresponding, Fndc5 protein levels were increased significantly by 230% and 130% on days 5 and 7 of differentiation, respectively, compared to day 0 (Figure 1E).

Similarly, in parallel to Fndc5 expression, Pgc-1α transcript levels were significantly increased by 6500% and 300% on day 5 and 7 of differentiation, respectively, compared to day 0. In addition, the transcript levels of Pgc-1α on day 5 were significantly higher than on day 7, by 210% (Figure 1F).

**Exogenic irisin effect on muscle cells and genes expression**

Given our findings that Fndc5 expression is differentiation-dependent, we further studied the effect of exogenous irisin during L6 cell differentiation (0-10 days). The treatment group received exogenous irisin (60 ng/ml) at the beginning of differentiation (day 0) and the end
Irisin regulates muscle differentiation and activity
Irisin regulates muscle differentiation and activity

Figure 1. Phenotype and molecular characterization of muscle L6 cells during differentiation. L6 muscle cells were grown and differentiated for 10 days using standard methods (materials and methods). The L6 cells were photographed under a light microscope (Olympus microscope IX81) at a magnification of 20X throughout differentiation. The analyses were performed using ImageJ software (1.46 version) software. Results were expressed as mean ± SE of 3 independent experiments (n=3). A. Visual differences in muscle fiber phenotype during the L6 differentiation. B. Mean muscle fiber thickness during L6 differentiation. C. Maximum muscle fiber thickness during L6 cell differentiation. L6 cells were differentiated and subject to mRNA extraction and protein isolation at different days of differentiation as indicated (materials and methods). Following cDNA isolation, Myogenin, Fndc5 and Pgc-1α transcript levels were analyzed by quantitative real time PCR (qRT-PCR) and normalized to transcript of housekeeping gene (S18). Proteins were subject to western analysis. D. Myogenin expression during L6 cells differentiation. E. Fndc5 expression levels during L6 cells differentiation. F. Pgc-1α expression levels during L6 cells differentiation. Results are expressed as mean ± SE of 5 independent experiments (n=3). Asterisks represent statistically significant difference *P<0.05, **P<0.01.

of differentiation (day 7). We studied both the phenotype and the molecular effect of exogenous irisin treatment.

Significant hypertrophy was demonstrated during L6 differentiation in control and irisin-treated groups (Figure 2A). Average muscle fiber thickness increased significantly with differentiation by 113%, 125%, and 150% on days 2, 5, and 10, respectively, compared to day 0 in the treatment group and by 114%, 118%, and 152% on days 5, 7, and 10, respectively compared to day 0 in the control group. The treatment group demonstrated a trend of elevated average muscle fiber thickness through differentiation compared to the control, however it did not reach significance (Figure 2B).

Treating L6 muscle cells with exogenous irisin at the early pre-differentiation stage (day 0) resulted in a significant elevation in Fndc5 transcripts levels, reaching a peak of 645% elevation on day 2 and of 375% increase on day 5. In contrast, exogenous irisin treatment at late L6 differentiation (day 7) resulted in significant reduction in Fndc5 transcripts levels by 230% at day 8 compared to the control group. This inhibitory effect of exogenic irisin treatment did not last past 48 hr, where Fndc5 levels returned to control levels (Figure 2C).
Irisin regulates muscle differentiation and activity

A

Day 0

Control

Treatment

Day 2

Day 5

Day 7

Day 10

B

C

Mean Fiber Thickness (μm)

Days

Days

FNDC5 mRNA level/S18
Irisin regulates muscle differentiation and activity

Taken together, irisin, suggestively through an autocrine mechanism, regulates muscle cells' *Fndc5* expression levels in a differentiation stage-dependent manner. While at early pre-differentiation state exogenic irisin induces long-term elevated *Fndc5* expression, at late differentiation exogenic irisin have a short-term inhibitory effect on *Fndc5* levels. Irisin treatment at the beginning of differentiation (day 1) significantly increased *Pgc-1α* transcripts levels on day 7 of differentiation. However, treatment with exogenous irisin at late differentiation (day 7) resulted in an immediate significant elevation in *Pgc-1α* transcripts levels by 200%, 190%, and 180% on days 7, 8, and 10, respectively, compared with the control group (Figure 2D). These results suggest that the effect of exogenous irisin on the expression of *Pgc-1α* is differentiation stage-dependent. It is noteworthy that at the late differentiation stage (days 7-10), exogenic irisin induces different autocrine effects on *Fndc5* and *Pgc-1α* expression.

**The effect of training type (aerobic and anaerobic) and muscle type (fast and slow) on Fndc5**

Many physiological and biomechanical factors influence contraction activity in skeletal muscles. In order to study the response of *Fndc5* to different types of training (anaerobic and aerobic) and in different muscles (fast and slow), we conducted a series of ex-vivo experiments using EPS and post-training molecular analysis.

In SOL muscle *Fndc5* mean transcripts levels were significantly higher by 300% than *Fndc5* transcripts levels in the EDL muscle, independent of training type (aerobic or anaerobic). The type of training (aerobic or anaerobic) did not alter *Fndc5* mean transcripts expression levels in both SOL and EDL muscles (Figure 3A, 3B).

In summary, these results demonstrate that the levels of *Fndc5* are affected by muscle types (fast and slow) but not affected by the type of exercise.

**HICT improves C57BL/6 mice’s physical performance**

To examine the efficacy of the training protocol, performance tests before and at the end of the training program were performed (Table 2). In sedentary (SED) mice, there was a statistically significant but limited increase in exercise tolerance (70%, *P*<0.01; Table 2). Improvement in
Irisin regulates muscle differentiation and activity

Figure 3. Fndc5 expression in different mice muscles following aerobic and anaerobic states. SOL and EDL muscles were surgically isolated from C57BL/6 male mice. The muscles were subject to different stimulations, generating aerobic and anaerobic states using stimulation electrical pulses system. After the different physical training (ex-vivo) the muscles were subject to total RNA extraction, following by reverse transcription to cDNA and qRT-PCR analysis. The results are expressed as mean ± SE of 3 independent experiments (n=14). A. Fndc5 expression levels in EDL muscle - aerobic activity (red circle full), anaerobic activity (red circle hollow), SOL muscle - aerobic activity (light blue circle full), anaerobic activity (light blue circle hollow). B. Levels of Fndc5 expression in the 2 different muscles after exercise, regardless of the type of activity (aerobic or anaerobic). Asterisks represent a statistically significant difference *P<0.05, ***P<0.001.

Table 2. High intensity training improve performance

<table>
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<td></td>
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<td>Post SED period</td>
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<tr>
<td></td>
<td>Pre HICT</td>
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<td>43±1</td>
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<td>Exercise Tolerance (min:sec)</td>
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<td></td>
<td>13:24±0:21</td>
<td>24:24±0:58**</td>
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SED - sedentary, HICT - High Intensity Continuous Training. Data are represented as mean ± SE. **P<0.01, ***P<0.001, as compared to pre-training period.

Exercise tolerance may be due to the age-dependent maturation of mice. HICT protocol significantly increased exercise tolerance by 85% compared to baseline performance (P<0.001), which is 15% more compared to the post sedentary period.

Effect of physical training on Fndc5 and Pgc-1α expression in various muscles of C57BL/6 male mice

Exercise training regulates different myokines, including irisin (although with some contradictory results). Physical activity involves orchestrated action of many types of muscles involved in different type of physical activity. One of the intriguing questions is whether different muscle types and activities regulate Fndc5. To answer this, we studied Fndc5 and Pgc-1α expression in different muscles and following different activity states in C57BL/6 male mice. We chose to select muscle types representing different categories; fast-twitch muscles; extensor digitorum longus (EDL), tibialis anterior (TA), GASTROCNEMIUS, and QUADRICEPS muscles, and slow-twitch muscle; soleus (SOL).

We found differential Fndc5 expression in transcript and protein levels in different muscles and training states (trained and untrained) (Figure 4B, 4C). The tolerance of trained mice increased significantly after training sessions (Figure 4A). We found a significant elevation in Fndc5 transcripts expression levels of 330%, 160%, and 440% in the SOL, EDL, and TA trained mice muscles, respectively, compared to levels in untrained respective mice muscles. Additionally, the SOL trained mice muscle Fndc5 expression levels were significantly elevated by 293%, 283%, 1087%, and 1182% compared to EDL, TA, GASTROCNEMIUS, and QUADRICEPS muscles, respectively. Similar results were found in untrained mice as well; Fndc5 expression levels were significantly elevated in the SOL muscle by 14098%, 382%, 298%, and 775% compared to EDL, TA, GASTROCNEMIUS, and QUADRICEPS muscles, respectively (Figure 4B).
Irisin regulates muscle differentiation and activity

The Fndc5 expression levels in trained mice SOL, EDL, TA, GASTROCNEMIUS, and QUADRICEPS muscles were significantly higher by 250%, 340%, 290%, 130%, and 120%, respectively, compared to the corresponding muscles in the untrained mice (Figure 4C).

In the untrained mice group, Fndc5 protein expression levels in the GASTROCNEMIUS muscle were significantly higher by 6032%, 3070%, and 1083%, and in the QUADRICEPS by 4140%, 2100%, and 742% compared to SOL, EDL, and TA muscles, respectively. Similarly, in the trained mice group the Fndc5 protein expression levels were significantly elevated in the GASTROCNEMIUS muscle by 3620%, 1225%, and 500% compared, and in QUADRICEPS muscle by 2284%, 774%, and 320% compared to SOL, EDL, and TA muscles, respectively (Figure 4C).

Figure 4. Expression levels of Fndc5 in five different muscles in trained and untrained mice. Five different muscles were surgically isolated from C57BL/6 trained and untrained male mice. The muscles were subject to total RNA extraction followed by reverse transcription to cDNA and qRT-PCR analysis. The muscle tissues were subject to protein isolation. Protein analysis was done by western blot using standard protocols (material and methods section). A. Tolerance assay of trained and untrained mice. B. Fndc5 mRNA expression levels in 5 different muscles of trained versus untrained mice. C. Fndc5 protein expression levels in 5 different muscles of trained versus untrained mice. The results are expressed as mean ± SE of 2 independent experiments, total mice (n=10), total muscles (n=100). Asterisks represent statistically significant difference between trained and untrained mice *P<0.05, ***P<0.001; number signs (#) represent statistically significant differences P<0.05 between muscles.
Irisin regulates muscle differentiation and activity

Fndc5 is a Pgc-1α dependent myokine; thus, we studied Pgc-1α expression levels in different muscles of trained and untrained mice. Overall, we found that Pgc-1α expression is muscle types and training dependent.

Pgc-1α transcripts expression levels were significantly higher by 580%, 380%, and 200% in the SOL, EDL, and TA muscles, respectively in trained mice compared to expression levels in untrained mice. Additionally, the EDL trained mice muscle Pgc-1α expression levels were significantly higher by 152%, 227%, and 430%, compared to TA, GASTROCNEMIU, and QUADRICEPS muscles, respectively. In untrained mice Pgc-1α expression levels are higher in the GASTROCNEMIU muscle by 549% and 338% compared to SOL and QUADRICEPS muscles, respectively (Figure 5A).

Similarly, to the Fndc5 protein expression levels, Pgc-1α mean protein expression levels were significantly higher by 190%, 220%, 160%, 160%, and 185%, in the SOL, EDL, TA, GASTROCNEMIU, and QUADRICEPS trained mouse muscles, respectively, compared to the levels in the corresponding untrained mouse muscles (Figure 5B). Additionally, Pgc-1α mean protein expression levels in the QUADRICEPS untrained mouse muscles were significantly higher by 835% compared to EDL muscle. Pgc-1α mean protein expression levels in the QUADRICEPS trained muscles were significantly higher by 578%, 700%, 330%, and 230% compared to SOL, EDL, TA, and GASTROCNEMIU muscles, respectively (Figure 5B).

Discussion

Exercise is known to have a beneficial effect on human health [30]. Normal muscle mass is associated with disease prevention, improvement of disease outcome, and human health [19, 30]. Muscle tissue is a metabolically active dynamic tissue that interacts with other tissues through secreted myokines, including irisin [31]. Irisin, a recently discovered myokine, has a broad metabolic activity, affecting adipose tissue metabolism [14-16]. Additionally, irisin...
levels are associated with and regulated by exercise and muscle hypertrophy [21-23, 28, 32]. However, irisin endocrine, paracrine and autocrine activity and its specific mechanism and regulation have not yet been elucidated.

To elucidate the role of irisin in muscle cell differentiation and function, we studied a standard and established model of skeletal muscle cells, L6 cells [33]. Through L6 differentiation, cells exhibited acquisition of typical phenotypic traits. L6 hypertrophy, expressed as fiber thickness and maximum fiber thickness, exhibited gradual and significant elevation throughout differentiation days, reaching a peak around days 7-10. Significant elevation in Myogenin expression was demonstrated in parallel to mature phenotype acquisition. This phenotype transformation follows studies showing that muscle cells differentiation is associated with a 10-20-fold increase in Myogenin [34, 35]. Fndc5 transcript and protein expression levels exhibited a transient expression pattern during L6 cell differentiation, peaking around day 5 of differentiation. This transient expression pattern corresponded to L6 cell hypertrophy and began to decline when hypertrophy reached a plateau. Our study shows, to the best of our knowledge, for the first time, that Fndc5 expression is associated with skeletal muscle L6 cells differentiation-induced-hypertrophy. Along the same lines, irisin injection to mice induced significant muscle hypertrophy, enhanced grip strength, and improved regeneration and hypertrophy through several suggested mechanisms, including activation of satellite cells and enhanced protein synthesis [6].

The irisin signal transduction pathway is mostly unknown; however, PGC1-α, a key regulator of several muscle cells genes and secreted myokines, is also known to regulate FNDCC5 [17, 20]. We show that the Pgc-1α expression pattern during L6 cells differentiation is identical to that of Fndc5, where Pgc-1α expression peaks at day 5 of differentiation. Our findings of the expression pattern of Pgc-1α in L6 are in accordance with a similar pattern found in C2C12 cells, where Pgc-1α expression levels peak around day 4 of differentiation [36]. This finding suggests that Pgc-1α is linked to the transient and unique Fndc5 expression during the differentiation of muscle cells.

The receptor for irisin has not been identified to date; however, initial work has shown a link between irisin autocrine effect on both young and mature muscle cells hypertrophy [36]. We show that exogenous irisin treatment increased muscle fiber thickness in the L6 model. Irisin treatment regulated muscle cells’ Fndc5 expression levels in a differentiation stage-dependent manner; while at early pre-differentiation state, exogenic irisin induces Fndc5 expression, at late differentiation, exogenic irisin inhibits Fndc5 expression. Furthermore, at an early pre-differentiation stage, exogenic irisin treatment prolonged Fndc5 expression in contrast to the short-term inhibitory effect on Fndc5 levels at late differentiation. It was shown that treating differentiated human primary muscle cells with exogenous irisin for short-term (6 and 24 h) results in down-regulation of Fndc5 (irisin) and Pgc-1α mRNA levels, suggesting irisin negative feedback loop to reduce its endogenous expression [37]. Our results reinforce this suggestive inhibitory feedback as demonstrated in differentiated L6 muscle cells. However, our findings show that contrary to the late differentiated stage, in non-differentiated L6 cells, the addition of irisin positively effect Fndc5 expression levels. Exogenous irisin treatment also regulated Pgc-1α expression in a differentiation-stage dependent manner. Pgc-1α is known to be up-regulated during myoblast differentiation, probably related to mitochondria development, and is dependent on several initial molecular events [36]. Thus, at the late differentiation stage (days 7-10), exogenous irisin induces different autocrine effects on Fndc5 and Pgc-1α expression. This could be due to direct or indirect and dependent or independent molecular pathways of both genes.

The challenge of studying irisin in the context of muscle tissue is the complexity of the tissue. Thus, we further investigated Fndc5’s role in muscle tissue in an ex-vivo model, which enabled us to study Fndc5 at the muscle level as an independent unit/tissue as opposed to the in-vitro cell level. Furthermore, the ex-vivo model allowed us to study both trained and untrained states in controlled conditions (temperature, duration of the activity, type of activity, etc.) and thus enabled better quantification of the different variables [38]. We studied acute aerobic and anaerobic (20 min) states using
electrical pulse stimulation on different isolated muscle types, SOL and EDL, which have typical and particular functions. The EDL muscle is predominantly fast [39], belonging to the group of physiological flexors, and the SOL muscle belongs to slow extensor muscles [40]. We found that the Fndc5 transcript expression levels in the SOL slow-twitch muscle were significantly higher than the transcript expression levels in the EDL fast-twitch muscle. Both aerobic and anaerobic activity did not alter Fndc5 transcript expression in EDL and SOL muscles. Using a similar methodology of exercise-mimicking treatment, additional study also failed to find Fndc5 mRNA and irisin secretion alternation in primary human myotubes [41]. Moreover, electrical pulse stimulation method was shown to have no effects on FNDC5 mRNA levels in in-vitro human skeletal muscle cell model [42]. Several studies have investigated the link between exercise and irisin serum levels. They have reported contradictory results, probably due to different methodology, with some studies demonstrating that irisin serum levels increase after an acute bout of aerobic exercise [43]. In contrast, other studies have failed to find any or minor changes in irisin serum levels after an anaerobic or acute bout of resistance exercise [44, 45] or between individuals undergoing aerobic interval training and sedentary individuals [46]. Notably, serum irisin levels were not found to have significant parallel changes in skeletal muscle FNDC5 expression [47, 48]. However, it has been shown that Fndc5 mRNA levels are higher in exercising muscles of mice than in non-exercising muscles. Similarly, FNDC5 mRNA levels were found to be higher in muscle samples taken from human individuals after a controlled period of an endurance exercise than in muscles of non-exercising individuals [46].

Physical activity involves orchestrated action of many muscles. One of the intriguing questions regarding irisin with exercise is whether there is differential regulation in different muscles. We demonstrate that Fndc5 expression is significantly higher in trained mouse muscles than in untrained ones. The novelty in our findings is that expression of Fndc5 was studied in five different muscle types: SOL, EDL, TA, GASTROCNEMIUS, and QUADRICEPS, representing both fast (white) and slow (red) fibers. In addition to selecting the different muscles according to the different categories of muscle types, we also chose to study the QUADRICEPS muscle, as previous studies have shown the clinical importance of QUADRICEPS in predicting functional outcomes following exercise [49]. Our findings show that Fndc5 transcript expression levels are muscle-type dependent in both trained and untrained states. Fndc5 transcript expression levels in SOL muscle (slow type) were significantly higher than to those found in fast-type muscles in both trained and untrained states. In addition, our findings show that Fndc5 expression levels in all trained mice’s muscles are significantly higher than in all untrained muscles. Correspondingly, Fndc5 protein expression levels were significantly higher in all trained mice’s different muscles compared to all untrained mice’s different muscles. However, as opposed to the higher Fndc5 transcript expression levels in the slow-type muscles, the Fndc5 protein levels were higher in fast-type muscles. These results suggestively indicate that irisin was secreted following synthesis in the slow-type muscles, resulting in low endogenous protein levels, or that post-translational modification resulted in lower protein levels. As indicated above, not in all cohorts and modes of exercise, positive associations were found between physical activity and plasma irisin levels in humans or animal models [15]. This could partly be due to the secretion of irisin to the local vicinity, as was demonstrated by immunohistochemical analysis [50]. Similar to irisin, PGC-1α is synthesized following exercise in muscles and stimulates several effects of exercise in muscles, including fiber-type switching, angiogenesis, and mitochondrial biogenesis [14, 51]. As Fndc5 was suggested to be a Pgc-1α dependent myokine, we studied Pgc-1α transcript levels in different muscles of trained and untrained mice. Similar to the expression of Fndc5 in muscles of trained versus untrained mice, we found that Pgc-1α expression levels in muscles of trained mice were significantly higher than levels in muscles of untrained mice. However, unlike Fndc5 expression, Pgc-1α expression levels were lower in slow-type (like SOL) muscles than in fast-type muscles (like EDL). Similar to our results, it was shown that type II (fast) fibers have a higher Pgc-1α level compared to type I fibers (slow), suggesting that each fiber type has its Pgc-1α baseline levels, which increase with exercise [51]. We demonstrated that Pgc-1α protein expression levels
Irisin regulates muscle differentiation and activity

were significantly higher in all five different muscles of trained mice compared to levels in muscles of untrained mice. Furthermore, we found that Pgc-1α protein expression levels were higher in fast muscle types: GAS-TROCNEMIUS (fast) and QUADRICEPS (slow and fast) compared to levels in slow muscle types.

In conclusion, we found that muscle cell’s Fndc5 expression has a differentiation-dependent pattern paralleling Pgc-1α expression and hypertrophy. Irisin autocrinally and significantly regulate Fndc5 and Pgc-1α in a differentiation-dependent manner. Muscle Fndc5 expression levels are dependent on fiber type and activity type.

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Disclosure of conflict of interest

None.

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Irisin regulates muscle differentiation and activity


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