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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 levels in 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

whole-body glycemic state (exercise-induced IL-6) [12, 13].

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 different 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% CO_2 at 37°C.

Muscle fiber thickness test

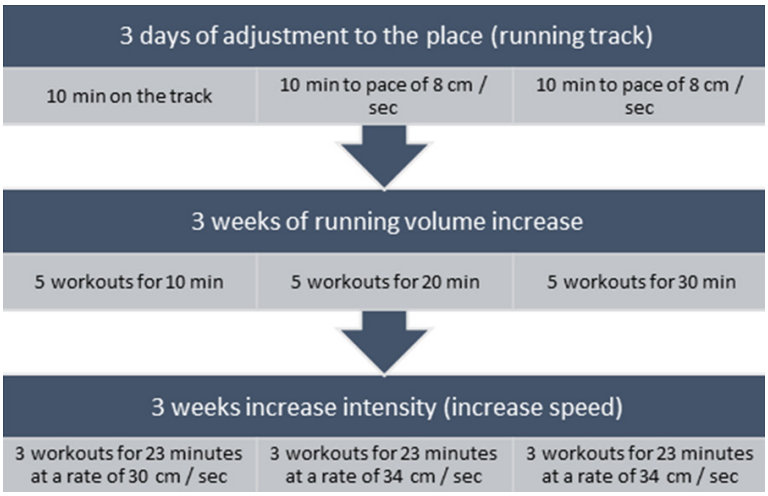
Cultured L6 cells (200,000 cells/ml) were seeded on 12 well plates, subjected to differentiation and photographed through days of differentiation, at time points: days 1-10. Photography was done using a light microscope (Olympus microscope IX81). Hypertrophy was evaluated by measuring the muscle fibers'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



Scheme 1. In vivo training protocol.

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_2PO_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.

Table 1. Primers used for qPCR

Mouse *Gapdh*	F-GTCTCCTCTGACTTCAACAGCG
	R-ACCACCCTGTTGCTGTAGCCAA
Mouse *Actin*	F-GCACCACACCTTCTACAATG
	R-TGCTTGCTGATCCACATCTG
Mouse *Fndc5*	F-ACAGGCAGAGAGCAGAGAGC
	R-GAAGTCTGCTGCCACATCAA
Mouse *Pgc-1α*	F-GGTGCCTTCAGTTCACTCTCA
	R-ACCAGAGCAGCACACTCTAT
Rat *S18*	F-GTAACCCGTTGAACCCCATT
	R-CCATCCAATCGGTAGTAGCG
Rat *Myogenine*	F-TGCACATCTGTTCGACTCTCTTC
	R-CCCTATCGTTCCCTCCCTTC
Rat *Fndc5*	F-AGAAGTCCATGCAACCAACC
	R-GGAGAACCTGTGGCTAGCTG
Rat *Pgc-1α*	F-CGCTTCCACCCAGATCACTT
	R-CTCTGTCCCCTCACAGGACT

RNA isolation

Total RNA was isolated using TRI reagent (Trizol, Rhenium, Israel) according to the manufacture 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 nM 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-

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 by 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. Intergroup 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].

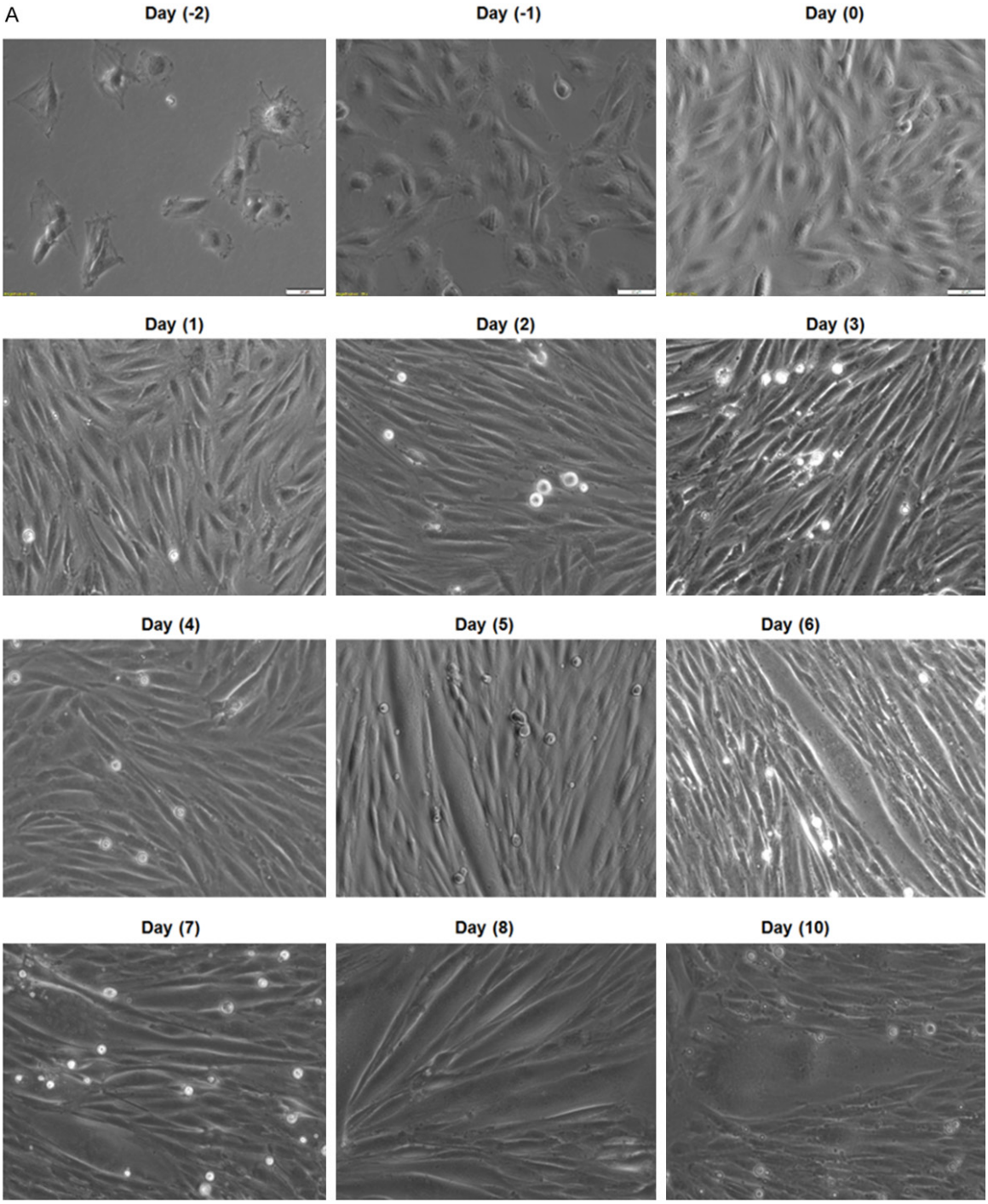
The morphology of undifferentiated pre-differentiation induction is demonstrated on days-2-0, where cells exhibit round shapes (**Figure 1A**). As differentiation was induced (day 1), cell morphology alterations were noted (**Figure 1A**). We found a significant increase in the mean thickness of the 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 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 increas zed 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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Mean Fiber Thickness (μm)
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Days

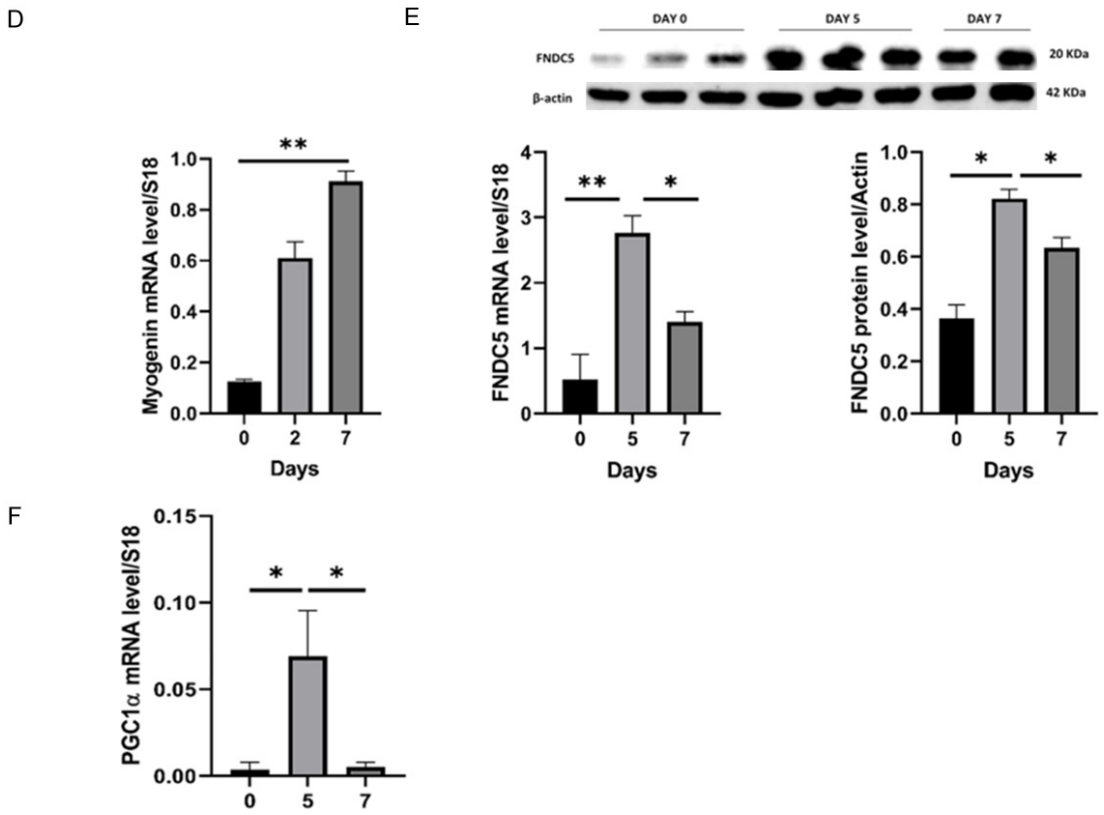
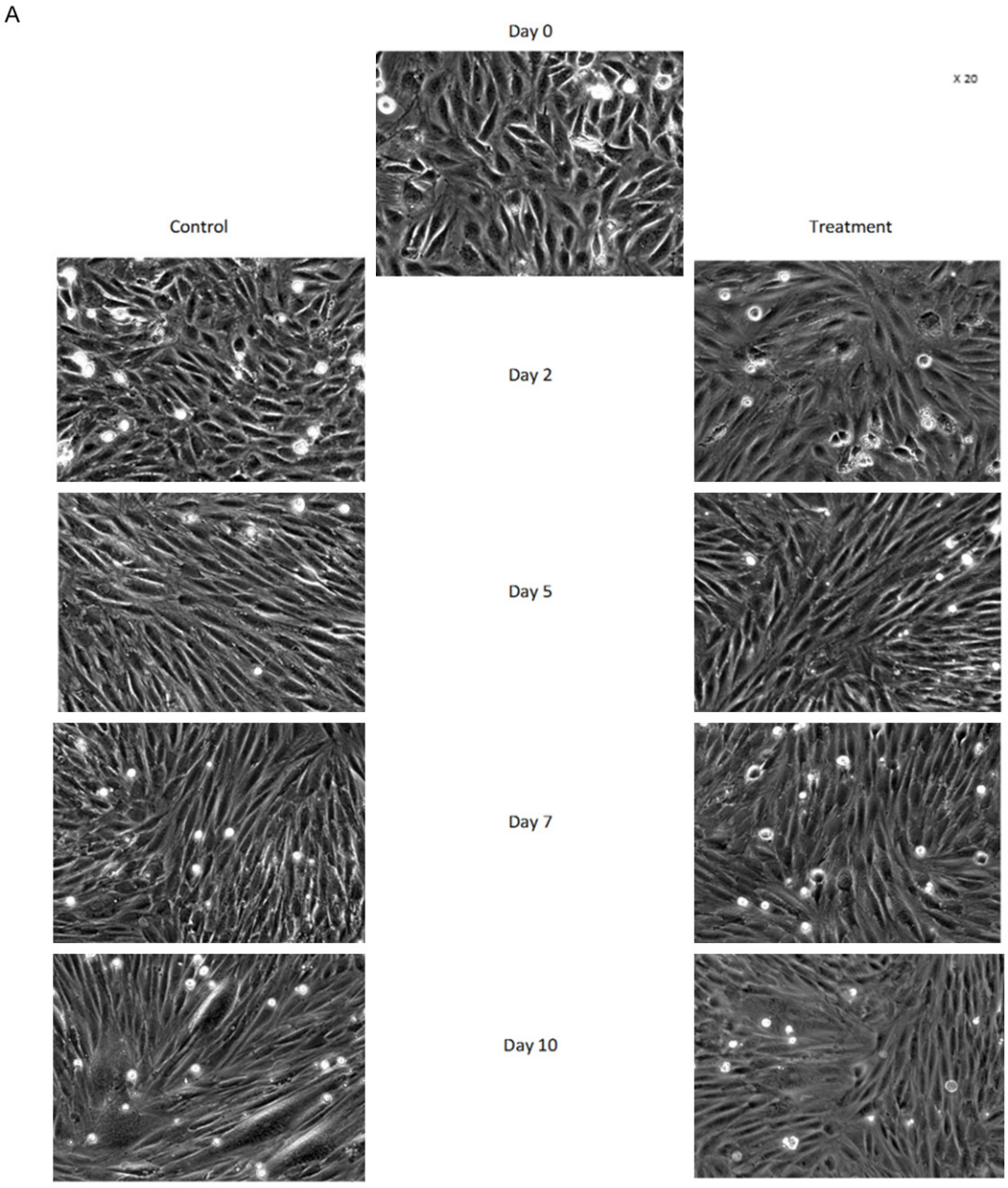


Figure 1. Phenotype and molecular characterization of muscle L6 cells during differentiation. L6 muscle cells were grown and differentiated for 10 days using standard methods (materials and methods). The L6 cells were photographed under a light microscope (Olympus microscope IX81) at a magnification 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**).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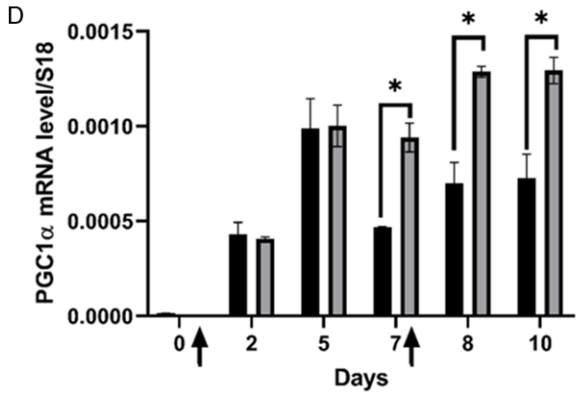

Figure 2. Exogenous irisin treatment during L6 muscle cells differentiation - phenotype and *Fndc5* and *Pgc-1α* expression. L6 muscle cells were grown and differentiated for 10 days. Treatment group received exogenous irisin (60 ng/ml) at the beginning of differentiation (day 0) and at the end of differentiation (day 7). Cells were photographed under a light microscope (Olympus microscope IX81) at a magnification of 20X. The analysis was performed in ImageJ software (1.46 version) software. A. Visualization of muscle fiber thickness during differentiation. B. Mean muscle fiber thickness during L6 cell differentiation. Cells were subject to complete mRNA extraction during differentiation days as indicated. Following cDNA isolation, *Fndc5* and *Pgc-1α* transcript levels were analyzed by qRT-PCR and normalized to transcript of housekeeping gene (*S18*). C. *Fndc5* expression following exogenous irisin treatment in treated and un-treated control groups. D. *Pgc-1α* expression following treatment with exogenous irisin in treated and un-treated control groups. Results are expressed as mean ± SE of 2 independent experiments (n=3). * Asterisks represent statistically significant difference (P<0.05); number signs (#) represent statistically significant differences (P<0.05) from day 0.

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

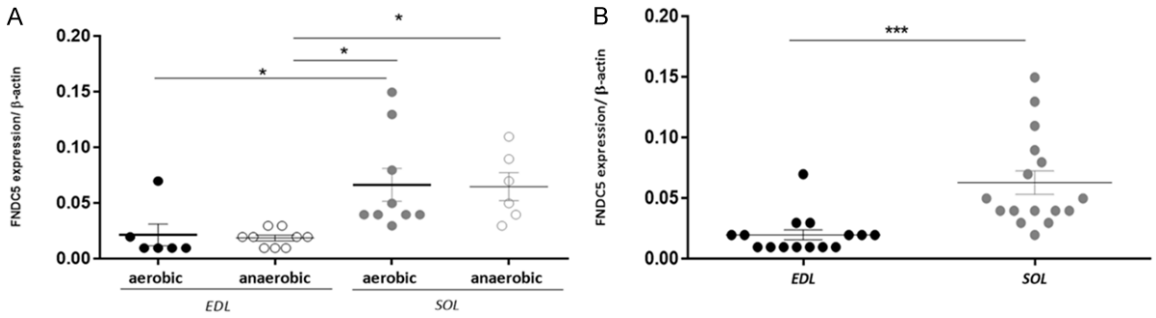

Figure 3. *Fndc5* expression in different mice muscles following aerobic and anaerobic states. *SOL* and *EDL* muscles were surgically isolated from C57BL/6 male mice. The muscles were subject to different stimulations, generating aerobic and anaerobic states using stimulation electrical pulses system. 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

Test type	SED (n=5)		HICT (n=5)	
	Pre SED period	Post SED period	Pre HICT	Post HICT
Exhaustion Speed (cm/sec)	46±2	43±1	44±2	46±1
Exercise Tolerance (min:sec)	13:24±0:42	22:03±0:55**	13:24±0:21	24:24±0:58***

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)*, *GASTROCNEMIU*, 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 respected mice muscles. Additionally, the *SOL* trained mice muscle *Fndc5* expression levels were significantly elevated by 293%, 283%, 1087%, and 1182% compared to *EDL*, *TA*, *GASTROCNEMIU*, 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*, *GASTROCNEMIU*, and *QUADRICEPS* muscles, respectively (**Figure 4B**).

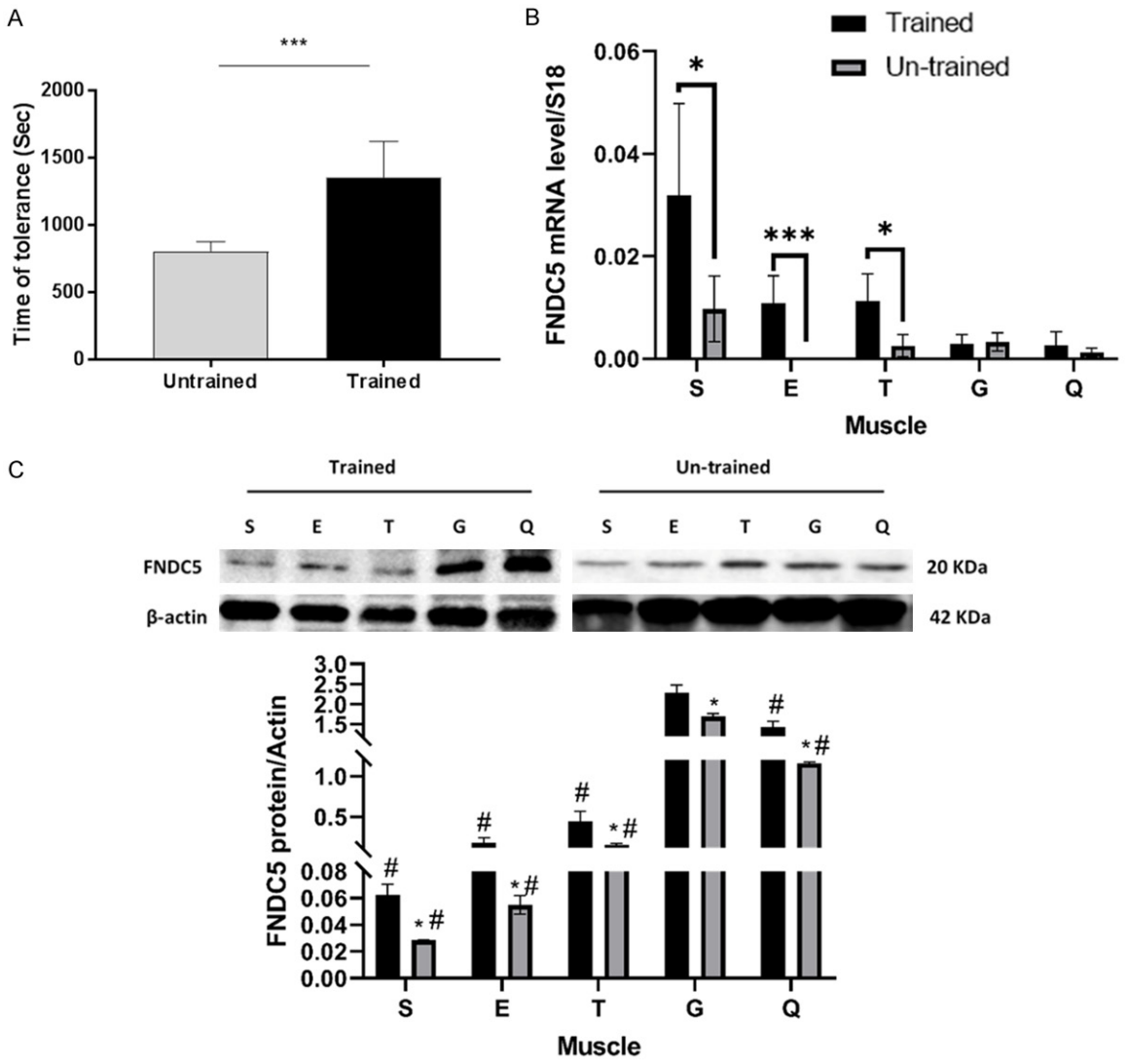

Figure 4. Expression levels of *Fndc5* in five different muscles in trained and untrained mice. Five different muscles were surgically isolated from C57BL/6 trained and untrained male mice. The muscles were subject to total RNA extraction followed by reverse transcription to cDNA and qRT-PCR analysis. 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.

The Fndc5 expression levels in trained mice *SOL*, *EDL*, *TA*, *GASTROCNEMIU*, 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**).

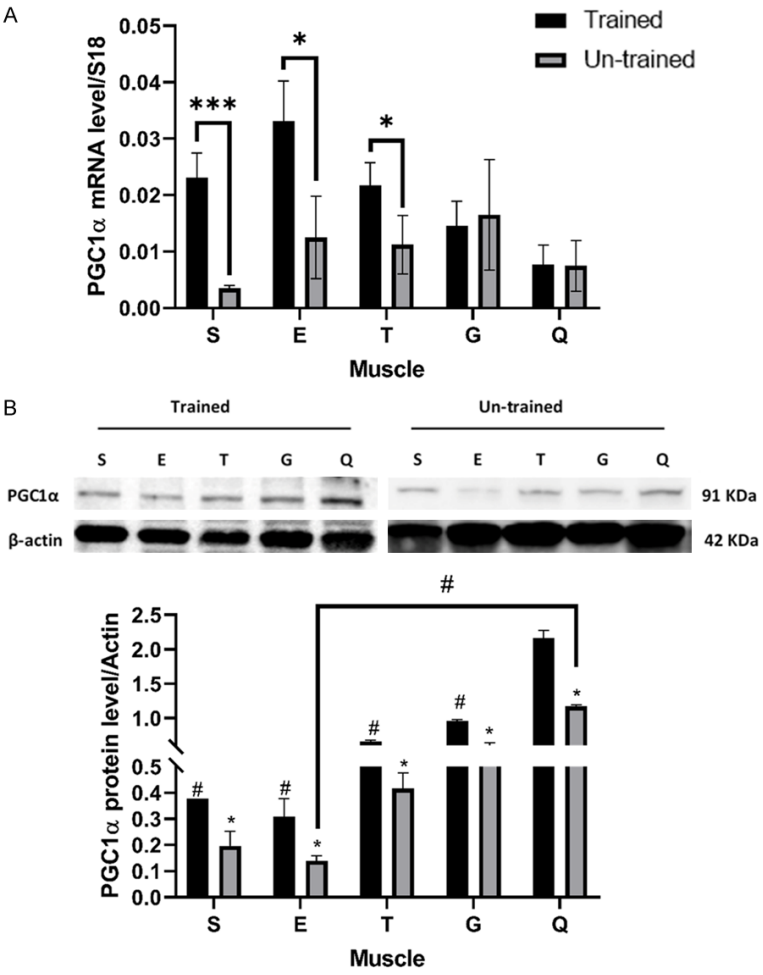

Figure 5. Expression levels of *Pgc-1α* in five different muscles in trained and untrained mice. Five different muscles were surgically isolated from C57BL/6 trained and untrained male mice. The muscles were subject to total RNA extraction followed by reverse transcription to cDNA and qRT-PCR analysis. The muscle tissues were subject to protein isolation. Protein analysis was done by western blot using standard protocols (material and methods section). A. *Pgc-1α* mRNA expression levels in 5 different muscles of trained versus untrained mice. B. *Pgc-1α* 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 from *QUADRICEPS* muscle.

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 mice muscles, respectively, compared to the levels in the corresponding untrained mice muscles (**Figure 5B**). Additionally, Pgc-1α mean protein expression levels in the *QUADRICEPS* untrained mice 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 acquirement 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 acquirement. 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 FNDC5 [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 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 endogenic 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

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: *GASTROCNEMIUS* (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-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.

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Disclosure of conflict of interest

None.

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