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The myokine decorin is regulated by contraction and involved in muscle hypertrophy

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ABSTRACT

The health-promoting effects of regular exercise are well known, and myokines may mediate some of these effects. The small leucine-rich proteoglycan decorin has been described as a myokine for some time. However, its regulation and impact on skeletal muscle has not been investigated in detail. In this study, we report decorin to be differentially expressed and released in response to muscle contraction using different approaches. Decorin is released from contracting human myotubes, and circulating decorin levels are increased in response to acute resistance exercise in humans. Moreover, decorin expression in skeletal muscle is increased in humans and mice after chronic training. Because decorin directly binds myostatin, a potent inhibitor of muscle growth, we investigated a potential function of decorin in the regulation of skeletal muscle growth. *In vivo* overexpression of decorin in murine skeletal muscle promoted expression of the pro-myogenic factor Mighty, which is negatively regulated by myostatin. We also found Myod1 and follistatin to be increased in response to decorin overexpression. Moreover, muscle-specific ubiquitin ligases atrogin1 and MuRF1, which are involved in atrophic pathways, were reduced by decorin overexpression. In summary, our findings suggest that decorin secreted from myotubes in response to exercise is involved in the regulation of muscle hypertrophy and hence could play a role in exercise-related restructuring processes of skeletal muscle.

1. Introduction

The beneficial effects of physical activity on health are well known, and increasing the amount of exercise have been shown to effectively protect against the development of many chronic diseases such as obesity and type 2 diabetes [1,2]. However, detailed mechanisms mediating the protective effects of physical activity remain poorly understood.

Skeletal muscle is recognized as a major endocrine organ releasing myokines that play a pivotal role in the communication between muscle and other tissues [3]. Thus, specific myokines released from contracting muscle might mediate health-promoting effects of physical activity [4]. Indeed, muscle metabolism is affected by a variety of myokines such as interleukin (IL)-6 and IL-15. In addition, myokines such as myostatin, follistatin and IL-7 are involved in the regulation of muscle mass in response to exercise (reviewed in [3]). An elevated muscle mass has beneficial effects by itself as seen in the case of sarcopenic-obesity, which carries a higher risk of type 2 diabetes [5].

Muscle contraction is supposed to be the main stimulus for the release of certain myokines. In order to identify novel myokines we have conducted a proteomics profiling of supernatants obtained from differentiated primary human skeletal muscle cells (SkMc)

Abbreviations: αMEM, α-modified Eagle's medium; EPS, electrical pulse stimulation; IL, interleukin; IVE, *in vivo* electroporation; SkMc, skeletal muscle cells; TGF, transforming growth factor; TA, *tibialis anterior*.

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and identified more than 500 myokines [6,7]. Comparison of these myokines with differentially expressed genes in a mouse exercise model revealed the small leucine-rich proteoglycan decorin as a secreted protein exhibiting increased expression in response to exercise.

Decorin is part of the extracellular matrix and is secreted by SkMc [8,9]. It plays an important role in cell growth through modulation of growth factor activities. Several reports have described a direct interaction of decorin with members of the transforming growth factor (TGF) beta family, which are thereby neutralized [10,11]. Of note, the TGF-beta family member myostatin is a strong inhibitor of muscle growth [12]. This study aimed to clarify the regulation of decorin release and expression in response to exercise and investigate a potential role of decorin for skeletal muscle growth.

2. Material and methods

2.1. Reagents

Reagents for SDS–PAGE were supplied by GE Healthcare Life Sciences (Freiburg, Germany) and Sigma (Munich, Germany). Human recombinant decorin was purchased from R&D Systems (Wiesbaden, Germany). The ELISA kit for decorin was delivered by RayBiotech (GA, USA). Gibco cell culture media and horse serum were supplied by Life Technologies (Darmstadt, Germany). Primary human SkMc and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). All other chemicals were of the highest analytical grade commercially available and purchased from Sigma.

2.2. Culture of primary human skeletal muscle cells and EPS treatment

Primary human SkMc of healthy donors (Caucasian, male, 16 years (M16) and 41 years (M41); female, 33 years (F33) and 37 years (F37)) were supplied as proliferating myoblasts and cultured according to the protocol of PromoCell as described before [13]. Myoblasts were seeded in six-well culture dishes and cultured in α-modified Eagle's (αMEM)/Ham's F-12 medium containing SkMc growth medium supplement pack up to near-confluence. Cells were then differentiated in αMEM containing 2% horse serum until day 5 of differentiation followed by overnight starvation in serum-free αMEM. Fresh serum-free αMEM was supplied directly before the stimulation. The myotubes were stimulated with a frequency of 1 Hz, a pulse duration of 2 ms and an intensity of 11.5 V for 6 h and 24 h. These EPS-conditions are optimized for maximal AMPK activation and IL-6 secretion [13]. Afterwards, supernatants of contracted and non-contracted SkMc were collected and analyzed by ELISA.

2.3. Animal studies

Animals were kept in a temperature-controlled room (22 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. All animal studies were conducted in accordance with the FELASA guidelines for the care and use of laboratory animals, and all experiments were approved by the ethics committee of the State Agency of Environment, Health and Consumer Protection (State of Brandenburg, Germany).

2.3.1. Exercise training

Mice were trained for 4 weeks (5 days/week) on a motorized treadmill (Exer6; Columbus Instruments; Columbus, USA), which was equipped with an electrical shock grid to encourage mice to complete the training sessions. To ensure a continuous training challenge a progressive training protocol, which increased its intensity over the 4 week training period, was used.

2.3.2. In vivo electroporation

In vivo electroporation (IVE) was performed as described previously [14]. In brief, plasmids were purified using Endotoxin-free Mega-Prep kits (Qiagen), and resuspended in sterile 0.9% saline. Animals were anesthetized with isofluran and their hind limbs shaved. *Tibialis anterior* (TA) muscles were injected with 15 units of hyaluronidase dissolved in sterile saline. After 60 min, animals were anesthetized again and 15 μg of decorin expression plasmid (pReceiver-M72-Decorin) was injected in the TA muscle. The contralateral muscle received injection with pEGFP-N1 vector (Clontech) as internal control. This was immediately followed by the application of a pair of tweezer electrodes (Harvard Apparatus, Holliston, USA) across the distal limb connected to an ECM-830 square wave electroporator (Harvard Apparatus, Holliston, USA). Application of eight 20 ms pulses of 80 V at a frequency of 1 Hz was used to facilitate plasmid uptake. Animals were sacrificed 7 days later and muscle rapidly dissected and shock frozen in liquid nitrogen.

2.4. Human exercise studies

2.4.1. Ethical approval

The studies adhered to the Declaration of Helsinki. All participants were informed about the project, procedures and rights before signing an agreement form. The acute study was approved by the National Regional Committee for Medical and Health Research Ethics, Region Sør-Øst-Norge, Norway. The intervention study was approved by the National Regional Committee for Medical and Health Research Ethics North, Tromsø, Oslo, Norway and registered with the US National Library of Medicine Clinical Trials registry (NCT01803568).

2.4.2. Human study I – acute resistance exercise

Ten well-trained healthy lean male volunteers (24.4 ± 0.6 kg/m^2) were recruited for the study. A baseline blood sample was taken before start of the exercise. A warm up was conducted by 5-7 min cycling on stationary bike. The strength training session was composed of seven exercises performed in three sets at a load corresponding to 8-RM (the weight that could be lifted maximal 8 times). The exercises were leg press, leg curls, bench press, pull-down, sitting shoulder press, cable-flies, and low rowing. A new set of exercise started every 3 min. Blood was sampled after the 3rd set of leg press, immediately after completion of the training session as well as 30 min, 60 min, 90 min and 120 min post exercise.

2.4.3. Human study II – 12 weeks exercise intervention study

Healthy and physically inactive men (40–65 years) were recruited in two groups; controls with normal weight (23.5 ± 2.0 kg/m^2) and normal fasting and 2 h serum glucose levels (n = 13) or overweight (29.0 ± 2.4 kg/m^2) with abnormal glucose metabolism (dysglycemic group, n = 13). Abnormal glucose metabolism was defined as fasting glucose $\geqslant$ 5.6 mmol/L and/or impaired glucose tolerance (2 h serum glucose $\geqslant$ 7.8 mmol/L). The participants were subjected to a combined strength and endurance training program for 12 weeks as described previously [15].

Biopsies from *m. vastus lateralis* were taken before as well as after 12 weeks of training and immediately transferred to RNA-later (Qiagen, Limburg, Netherland), kept overnight at 4 °C and transferred to −80 °C.

2.5. Analysis of plasma samples

All plasma samples were kept on ice until centrifugation and were stored at −70 °C afterwards until analyses were performed. Decorin concentration was assessed using a human decorin ELISA according to the manufacturer's protocol.

2.6. Expression analyses by quantitative real time PCR

RNA was isolated from mouse muscle samples according to the manufacturer's instructions using TRI-reagent (Sigma Aldrich). For conversion into cDNA random primers and the cDNA synthesis kit from Qiagen were used. Quantitative real time PCR was performed on Roche Lightcycler 480 using gene specific primers and corresponding UPL Probes (Roche). The following targets were measured with commercially available TaqMan assays (Applied Biosystems): decorin, myostatin, Mighty, follistatin, Myod1, atrogin1, and MuRF1. The eukaryotic translation elongation factor 2 (Eef2) was used as endogenous control.

2.7. High throughput mRNA sequencing

Frozen human muscle biopsies were crushed to powder in a liquid nitrogen-cooled mortar using a pestle. QIAzol Lysis Reagent (Qiagen) was added to muscle tissue powder and the samples were homogenized using TissueRuptor (Qiagen). Total RNA was then isolated by miRNeasy Mini Kit (Qiagen).

All mRNA samples were deep sequenced using the Illumina HiSeq 2000 system with multiplexed design. Illumina HiSeq RTA (real-time analysis) v1.17.21.3 was used for real-time analysis during the sequencing run. Reads passing Illumina's recommended parameters were demultiplexed using CASAVA v1.8.2. For pre-alignment quality checks we used the software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The mean library size was 44.1 million unstranded single-ended reads with no difference between groups or time points. No batch effects are present. Reads alignment was done using Tophat v2.0.8 [16], Samtools v0.1.18 [17] and Bowtie v2.1.0 [18] with default settings against the UCSC hg19 annotated transcriptome and genome dated 14th of May 2013. Post-alignment quality checks were done using the Integrative Genome Viewer 2.3 [19,20] and BEDtools v2.19.1 [21]. Reads counted by gene feature were done using the intersection strict mode in HTSeq 0.6.1 [22].

2.8. Statistics

Statistical evaluation was done by Student's *t*-tests for paired or unpaired observations and one-way ANOVA (post hoc test Tukey's multiple comparison test). For differential expression analysis of decorin using RNA sequencing, edgeR v3.4.2 [23] was used to calculate statistical significance. A p-value of <0.05 was considered as statistically significant. Normalized gene expression levels are presented in fragments per kB mapped reads (FPKM). Filtering strategies, quality checks and generalized linear model construction were done in R v3.0.3 following the edgeR developers' recommendations (http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf). Correlation analyses were performed in SPSS v20.0 and Prism6 (GraphPad, LA Jolla, CA). Pearson's correlation coefficient was used on normal distributed parameters. Spearman's rank correlation was used on non-normal parameters and on analyses including few samples. Data are presented as means ± SEM.

3. Results

3.1. Regulation of decorin by contraction in vitro and in vivo

Differentiated primary human myotubes were exposed to EPS resulting in visible contraction [13]. Analyzing the supernatant of contracting vs. non-contracting myotubes by ELISA revealed a significantly increased release of decorin in response to 24 h EPS (Fig. 1A). Moreover, decorin mRNA expression was significantly increased in skeletal muscle samples of mice that underwent four weeks of progressive exercise training in comparison to untrained control mice (Fig. 1B).

3.2. Regulation of decorin by exercise in humans

In order to validate that our findings in cell culture and exercised mice are relevant for humans, we measured plasma decorin levels in healthy lean volunteers who underwent a resistance exercise challenge. As shown in Fig. 2A, plasma decorin levels were significantly increased at the end of the exercise session. Afterwards, the levels returned to baseline levels. Moreover, we found a positive correlation of decorin levels immediately after the exercise session with the strength to perform the leg press exercise. Volunteers who could push more weight also displayed higher a increase of decorin levels from baseline to the end of the training session (Fig. 1B).

We also assessed expression changes of decorin in human skeletal muscle after a 12 weeks training intervention that combined endurance and resistance exercise. As shown in Fig. 3A, no difference between basal levels of decorin expression in skeletal muscle of control vs. dysglycemic subjects was found. Interestingly, we observed a positive correlation between decorin expression and fat free mass before the intervention (Fig. 3B). Analyses of decorin expression after the intervention revealed a significant increase of decorin expression in control subjects whereas in the dysglycemic group a trend towards reduced decorin expression was found (Fig. 3A). Moreover, in control subjects a positive correlation was found between increased decorin expression and increased leg press strength after the intervention (Fig. 3C).

3.3. Effect of decorin overexpression on skeletal muscle hypertrophic pathways

Because decorin can antagonize myostatin we assessed a possible function of decorin in muscle hypertrophic pathways. IVE-induced overexpression of decorin resulted in a 9.7-fold increase of its expression compared to GFP-control (Fig. 4A). Expression of Mighty, which is negatively regulated by myostatin, was significantly increased (∼1.3-fold) in decorin-overexpressing TA muscle, but also myostatin expression itself was increased ∼1.2-fold (Fig. 4B). Additionally, we found a slight but significant increase in the expression of Myod1 and follistatin, which are both involved in the regulation of muscle growth (Fig. 4C).

Furthermore, we measured expression of atrogin1 and MuRF1, which are involved in muscle atrophy. For both targets, we found significantly reduced levels (∼30%) in decorin-overexpressing TA muscles (Fig. 4D).

4. Discussion

The proteoglycan decorin has been known as a myokine for some time [9] but so far most studies investigated decorin only in tendons. Data regarding the regulation of decorin in skeletal muscle are scarce. In this study, we report enhanced secretion from

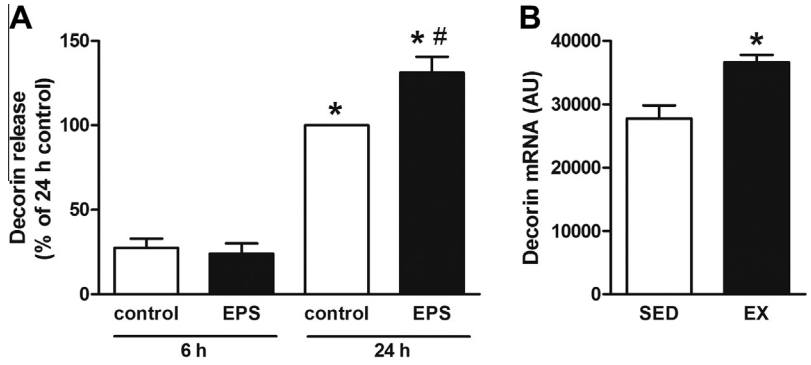

Fig. 1. Regulation of decorin by contraction *in vitro* and *in vivo*. (A) Differentiated primary human SkMc were treated with EPS for 6 h and 24 h or left unstimulated (control). Decorin concentration in supernatants was analyzed by ELISA. Data are shown relative to 24 h control and represent means ± SEM, $n \geqslant 6$, $^{*}p < 0.05$ vs. 6 h, $^{\#}p < 0.05$ vs. 24 h control (ANOVA). (B) Male C57BL/6J mice were endurance-trained on a motorized treadmill. Decorin mRNA expression in skeletal muscle was determined 2 h after the last exercise bout and compared to sedentary control animals. Data represent means ± SEM, $n = 6$, $^{*}p < 0.05$ (Student t test).

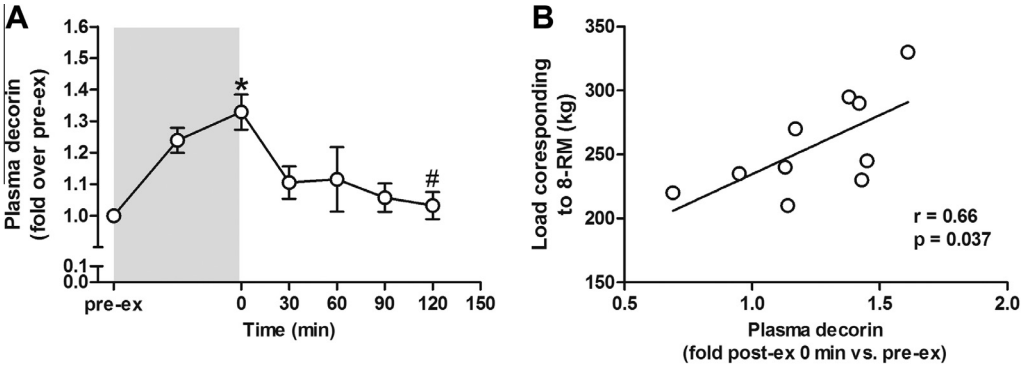

Fig. 2. Regulation of human plasma decorin levels by exercise. (A) Kinetic of plasma decorin levels in response to acute resistance exercise. Blood samples were taken before start of the exercise (pre-ex), after the third set of leg press, immediately after the exercise session (0 min) as well as 30 min, 60 min, 90 min, and 120 min post exercise. Data are shown relative to pre-ex and represent means ± SEM, $n = 10$, $^{*}p < 0.05$ vs. pre-ex, $^{\#}p < 0.05$ vs. post-ex 0 min (ANOVA). (B) Correlation of plasma decorin increase (post-ex 0 min vs. pre-ex) with load corresponding to 8-RM of leg press exercise. The r correlation coefficient and p-value was obtained by Pearson's test.

in vitro contracting human myotubes as well as increased decorin expression in murine skeletal muscle in response to chronic endurance training. Moreover, we show for the first time that plasma levels of decorin in human volunteers increase in response to acute resistance exercise. We have analyzed decorin expression in skeletal muscle after a 12 weeks training intervention and observed elevated mRNA of decorin in healthy control subjects. In accordance with our results, another study reported increased decorin expression in human skeletal muscle 6 h after completion of an acute endurance-type challenge using 1 h one-leg kicking as exercise model [24]. Although no other reports are available concerning circulating decorin after exercise our data suggest that decorin is a myokine, which is regulated by and secreted during exercise in skeletal muscle.

Proteoglycans are extracellular matrix components and modulators of growth factor activities. Decorin is known to antagonize myostatin [10], which is the most powerful inhibitor of muscle growth known [12]. Kishioka et al. demonstrated that decorin enhances proliferation and differentiation of C2C12 myoblasts by suppressing the activity of myostatin [11]. Because exercise induces muscle growth, we investigated effects of IVE-induced decorin overexpression in skeletal muscle on several pathways involved in regulation of muscle mass. First, we indirectly assessed consequences on the transcriptional activity of myostatin by analyzing the expression of the unique myostatin target gene Mighty [25], which was significantly enhanced. Marshall et al. reported a ~60% increased Mighty expression in skeletal muscle of myostatin-null mice whereas Mighty expression in C2C12 myoblasts was reduced by myostatin in a dose-dependent manner [25]. Their data suggest that the Mighty gene is negatively regulated by myostatin and that Mighty expression presents a reliable readout for the transcriptional activity of myostatin in skeletal muscle cells [25,26]. Thus, our data showing increased Mighty expression after decorin overexpression point towards a reduced transcriptional activity of myostatin. Because decorin can interact with myostatin thereby suppressing its activity, we suggest that our observation is a consequence of elevated decorin level [10,11]. Interestingly, we observed also an increase of myostatin expression, which might suggest a counter regulatory increase in response to inhibition of the transcriptional activity of myostatin.

Another potent regulator of skeletal muscle growth is follistatin, which can directly antagonize myostatin action [27]. In our model of *in vivo* decorin overexpression we found a slightly but significantly increased mRNA level of follistatin. Accordingly, in C2C12 myoblasts decorin treatment was shown to increase follistatin protein [28]. This effect of decorin would add to the inhibition of myostatin.

The myogenic factor Myod1 is one of the major regulators of myogenesis [29]. Several pathways affect expression of Myod1: (I) inhibition by myostatin [30], (II) activation by follistatin [27], and (III) induction by Mighty overexpression [25]. We found Myod1 expression to be significantly enhanced in response to decorin overexpression suggesting that the muscle proliferation/differentiation system is activated.

Muscle growth is not only a consequence of activated anabolic pathways but also of catabolic processes. Thus, we also investigated two effectors of muscle catalysis. The muscle-specific E3 ubiquitin ligases atrogin1 and MuRF1 execute their effects in the

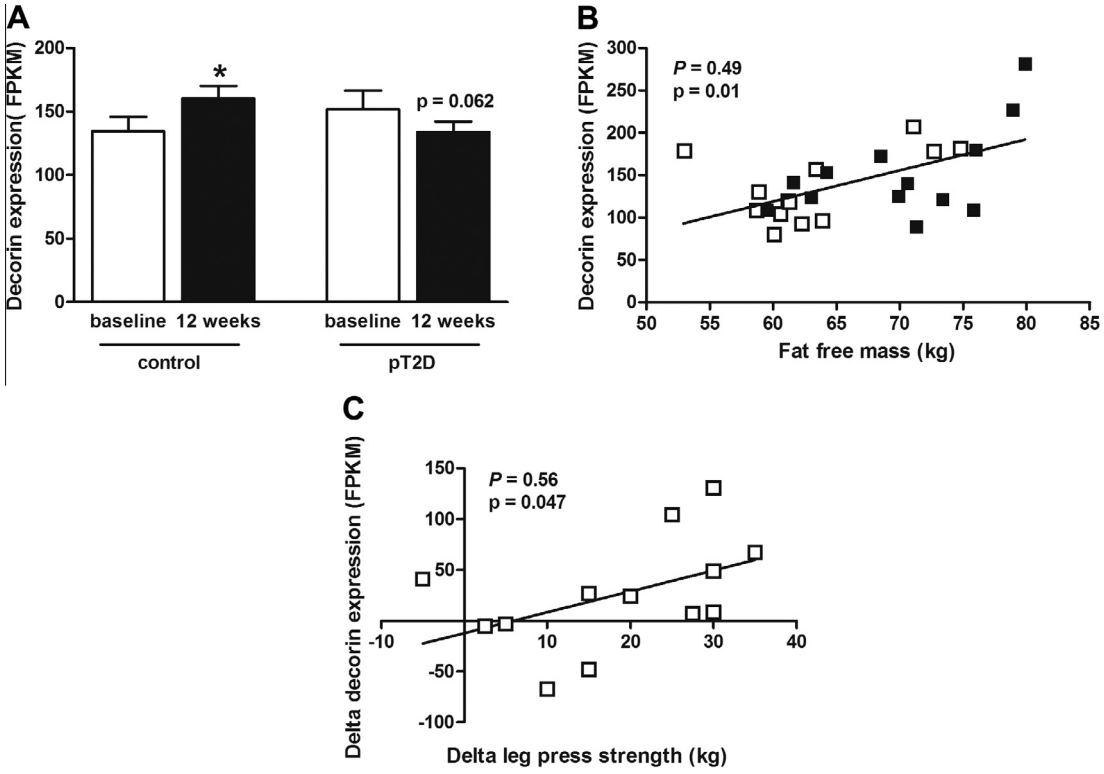

Fig. 3. Decorin mRNA expression in skeletal muscle after 12 weeks training intervention. (A) Decorin expression in *m. vastus lateralis* was assessed in healthy controls (white bars) and dysglycemic subjects (pT2D, black bars) before and after the intervention period. (B) Correlation of decorin expression with fat free mass in both groups before the intervention, $n = 26$. (C) Correlation of change of decorin expression with change in leg press strength in healthy controls during intervention ($n = 13$). The *P* correlation coefficients and *p*-values were obtained using Spearman's ranked correlation.

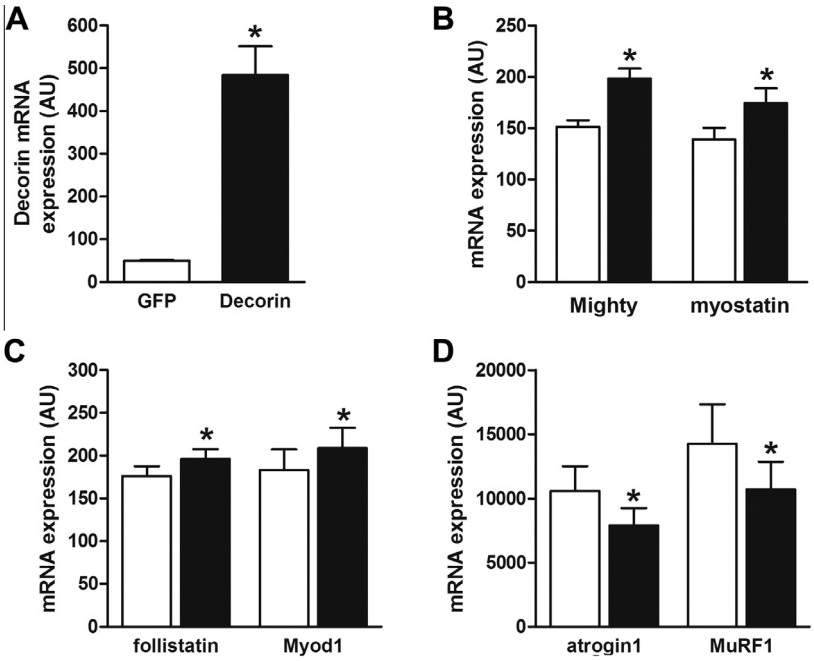

Fig. 4. Decorin overexpression and impact on gene expression related to muscle hypertrophy (A) IVE was used to overexpress decorin in TA muscle of mice resulting in a 9.7-fold increased decorin expression. IVE induced significantly increased expression of myostatin and Mighty (B) as well as follistatin and Myod1 (C). (D) Expression of atrogin1 and MuRF1 was significantly reduced upon decorin overexpression. Data are normalized to Eef2 and represent means ± SEM, $n = 26$, $^{*}p < 0.05$ vs. GFP-control (paired Students *t* test).

ubiquitin proteasome pathway, the primary pathway for intracellular protein degradation in skeletal muscle [31]. Both ubiquitin ligases exhibit a reduced expression in response to exercise [32]. The lower expression of atrogin1 and MuRF1 in response to decorin overexpression suggests that this catabolic pathway is less active, which would enhance the rate of skeletal muscle growth.

In summary, our study establishes decorin as an exercise-regulated myokine that is secreted in response to muscle

contraction. Following *in vivo* overexpression of decorin we found increased expression of different genes, which are involved in pathways of skeletal muscle growth. This was accompanied by reduced catabolic processes often suppressed during muscle growth. Consistent with a putative function in muscle hypertrophy enhanced levels of plasma decorin were observed in response to hypertrophy-promoting strength exercise. Thus, we hypothesize that decorin secreted from skeletal muscle cells in response to exercise is involved in restructuring of muscle during hypertrophy.

Conflict of interest/disclosure

The authors have no conflict of interests in relation to the contents of this paper.

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