Galectin-3 regulates skeletal muscle loss via TGFβ1-Smad2/3 signaling activation in mice

Keywords
galectin-3, skeletal muscle, glucocorticoids, TGFβ1, Smad2/3

Abstract

Introduction

Endogenous glucocorticoids (GCs) played a pivotal role in the pathogenesis of skeletal muscle loss. However, to date, the underlying molecular mechanisms underlying are not yet fully understood. Galectin-3 (Gal-3) is a member of a beta-galactoside-binding animal lectins, consistently associated with inflammation and fibrosis in the pathogenesis of various disease states. The present study aimed to explore the role of Gal-3 in GC-induced skeletal muscle loss.

Material and methods

Myogenic differentiation capacity was detected after in vitro Gal-3 knockdown (KD) or in vivo administration of Gal-3 inhibitor. The activation of transforming growth factor type beta 1 (TGFβ1) and Smad2/3 signaling pathways was determined by western blot, co-immunoprecipitation, and immunofluorescence.

Results

Gal-3 was up-regulated during dexamethasone (Dex) administration in mice. In the established GC-induced muscle loss model, Gal-3 inhibition recovered grip strength and muscle mass. In vitro, Gal-3 KD promoted the myogenic differentiation capacity of C2C12 myoblasts, and prevented the reduction of fully differentiated myotubes. Gal-3 results in overexpression of TGFβ1 and TGFβR-I, which affects Smad2, 3 phosphorylation and subsequently mediates skeletal muscle reduction by activating the Smad2/3 signaling pathways.

Conclusions

The present study demonstrated that Dex elevated Gal-3 levels in skeletal muscle. Gal-3 facilitates the activation of TGFβ1-Smad2/3 signaling pathways in myoblasts, and contributes to myogenesis inhibition and skeletal muscle loss. This study raises awareness about the follow-up of patients receiving GC therapy. Further, inhibition of Gal-3 provides a possible therapeutic strategy for skeletal muscle loss.

Title: Galectin-3 regulates skeletal muscle loss via TGFβ1-Smad2/3 signaling activation in mice

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Running head: Effects of Galectin-3 on mice skeletal muscle

Abstract

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1. Introduction

Nowadays, musculoskeletal disorders have become the leading cause of years lived with disability (YLDs) globally [1]. Previous studies have suggested that many pathological conditions characterized by muscle loss are associated with increased circulating GC levels [2,3]. Compared to a comprehensive understanding of the adverse outcomes, the complex mechanisms and pathways underlying these disorders are not yet fully understood.

Skeletal muscle growth and loss occur in a balanced and coordinated manner in order to maintain muscle mass and shape largely unchanged throughout adult life. Muscle stem cells, termed as satellite cells (SCs), are directly responsible for the regeneration capacity of skeletal muscle. The formation of skeletal muscle, i.e., involves a cascade of muscle-specific genes expressed programmatically and sequentially. A fundamental step in this process is the upregulation of myogenic regulatory factors (MRFs), including transcription factors, such as myogenic factor 5 (Myf5), myoblast determination protein (MyoD), myogenin (MyOG) and muscle specific regulatory factor 4 (Mrf4) [4-6]. The upstream signals that activate MRFs differ significantly at various physiopathological stages, although some molecular cues are shared; additional studies are required to address this concern.

Galectin-3 (Gal-3), also known as Mac-2, is a β-galactoside binding lectin, expressed in various tissues and participating in several physiological and pathological processes [7]. A number of previous studies have shown that Gal-3 is a major mediator of exacerbating disease in inflammatory, metabolic, and malignant diseases [8-9]. Specifically, Gal-3 has been closely related to myofibroblast activation, cardiac fibroblasts proliferation, hepatic progenitor cells expansion, and bone cells differentiation/maturation [10-13]. The link between Gal-3 and these pathological conditions lies in the modulation of cell proliferation and differentiation. Our previous studies have demonstrated that GC stress upregulated the level of secretory Gal-3 [14]. However, little is known about the function of Gal-3 in skeletal muscle biology.

In the present study, we found that Gal-3 is overexpressed in the GC-induced muscular loss model. The effects of Gal-3 on muscle loss were examined in an *in vivo* murine model with Gal-3 inhibitor-TD139 administration and an *in vitro* myoblasts model transfected with siRNA of Gal-3. Also, the involvement of TGFβ1/Smad2/3 signaling was explored in the anti-myogenic effects of Gal-3, aiming to explore the role and mechanism of Gal-3 in GC-induced skeletal muscle loss.

2. Material and Methods

2.1 Animal experiments

Male C57BL/6 mice (n=25, 8-weeks-old) were purchased from Shanghai Model Organisms Center (Shanghai, China). For GC-induced muscle loss model, 12weeks old C57BL/6 mice (n=25) were treated with either dexamethasone (Dex) (n=20) 25 mg/kg/day (D1756, Sigma) or an equivalent volume of normal saline (n=5) via intraperitoneal injections (i.p.) for 10 days. For TD139 (HY-19940, MedChemExpress) administration (n=10), TD139 was administered i.p. at a dosage of 15 mg/kg/day during the last five days of Dex treatment. The grip strength of each mouse was measured three times separately by a rodent grip strength meter (YLS-13A, Jinan Yiyan Company, China). The maximum value represented the grip strength of each mouse. All animal studies were reviewed and approved by the ethics committees of the School of Life Science of Fudan University (Approval No. 202101004S).

2.2 Tissue collection

All mice fasted for 4 h (from 08:00 a.m. to 12:00 a.m.) before body composition analysis by an animal whole-body composition analyzer (EchoMRI, USA). After euthanasia, the hindlimb muscles, including tibialis anterior (TA), gastrocnemius (GAS), and quadriceps (QUAD), were harvested and either fixed in 4% paraformaldehyde (PFA) or snap-frozen and stored at − 80℃ for further analysis.

2.3 Cell culture and myogenesis

C2C12 mice myoblast cells were purchased from Fuheng Cell Center (Shanghai, China) and cultured as described previously [15]. To initiate myogenesis *in vitro*, the proliferating C2C12 myoblasts were

shifted from growth medium (GM) into differentiation medium (DM) and harvested at the indicated time points. 50 μM Dex (D1756, Sigma) and/or 10 μM TD139 (HY-19940, MedChemExpress) was added into DM as required.

2.4 Galectin-3 siRNA transfection

C2C12 transfection was performed with Lipofectamine RNAiMAX transfection reagent (Invitrogen, USA). The transfection of siRNAs for Gal-3 (Hanbio Biotechnology Co., China) was performed before myogenesis was initiated, and after 24 h, cells were further cultured in DM and harvested at the indicated time points.

2.5 Staining and imaging

Immunofluorescence (IF) staining was performed as described previously [16]. Related primary antibodies included anti-MYHC II (1:400, M4276, Sigma) and anti-Smad2/3 (1:200, 8685, CST) antibodies. Mounted slides were imaged under a LEICA TCS SP8 confocal microscope (Leica, Germany) or fluorescence microscope (Leica, Germany) and the images were analyzed by Image J.

2.6 Western blot assay

Western blotting analysis was performed as reported previously [17]. We used RIPA lysis buffer (Beyotime, China) containing Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) to extract proteins from C2C12 cells or muscles. The protein concentration was determined using a BCA protein assays kit (23225, Thermo Scientific). The primary antibodies included anti-MYHC I (1:1000, BA-F8, DSHB), anti-MYHC II (1:1000, M4276, Sigma), anti-MyoD (1:200, sc-377460, Santa Cruz), anti-Myogenin (1:250, ab1835, Abcam), anti-TGFβR-I (1:1000, ab31013, Abcam), anti-Galectin 3 (1:1000, ab76245, Abcam), and Smad2/3 Antibody Sampler Kit (1:1000, 12747, CST). The protein expression was detected by the enhanced chemiluminescent method and imaged on a Molecular Imager System (Bio-Rad, USA). The protein levels were determined using MetaMorph software (Image J, USA).

2.7 Co-immunoprecipitation (Co-IP)

C2C12 cells were harvested after 2 days of culture in diameter 10-cm dishes, digested with 500μL IP lysis buffer, and the supernatant was collected by centrifugation at 12000 ×*g*, 4 °C for 20 min to obtain supernatant, of which 50 μL was collected as input samples. As a negative control (NC), the remaining of the digested solution was mixed with 5 μL normal rabbit IgG and 10 μL protein A/G agarose beads. For IP test, the remaining supernatant was incubated with 10 μL anti-TGFβR-I (ab31013, Abcam) and 10 μL protein A/G agarose beads. After centrifugation at 12000 ×*g*, 4 °C for 5 min and three washes in IP lysis buffer, the pellet was resuspended in 30 μL of 1X SDS solution. The immunoblots were probed with anti-Smad3 mouse monoclonal antibody (1:1000, 9513s, CST).

2.8 RNA extraction, reverse transcription, and qRT-PCR

qRT-PCR analysis was performed as reported previously [16]. Total RNA was extracted from C2C12 cell lines using an RNA extraction kit (Vazyme Biotech, China). Skeletal muscles were solubilized in TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA extracted from cells and muscle tissues was reverse transcribed into cDNA using TaKaRa SYBR Premix Ex Taq^{TM} and subsequently underwent quantitative real-time PCR utilizing StepOnePlus Real-time PCR Instrument (Applied Biosystems, USA). The expression of the target genes was normalized to that of *GAPDH*. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.9 Statistics.

Data between two groups were statistically analyzed using unpaired, two-tailed Student's t-test. All analyses were performed using GraphPad Prism 6.0. The results are presented as mean ± standard deviation (SD).

3. Results

3.1 Gal-3 was Upregulated in GC-induced Skeletal Muscle Loss Model

In GC-induced skeletal muscle, Gal-3 is highly expressed at both the gene and protein levels (Figure 1A–C). Also, the protein levels of fast myosin heavy chains (MyHC-II) and slow myosin heavy chains (MyHC-I) were decreased in the gastrocnemius muscles of the Dex group (Figure 1A, 1B).

Consistent with this finding, Gal-3 level was increased with the increasing of Dex concentrations in differentiated C2C12 myotubes (Figure 1D–F). Together, these data suggested that Gal-3 is ubiquitously upregulated in GC-induced muscle loss models both *in vitro* and *in vivo*.

3.2 Gal-3 Gene Knock-down (KD) Promoted Myogenic Differentiation in C2C12 Cells

Gal-3 KD significantly suppressed Dex-induced increase in Gal-3 (Figure 2A, 2B) and blocked the Dex-induced alteration of cell morphology in C2C12 myotubes, accompanied by a significantly enlarged diameter and number of differentiated myotubes (Figure 2C–E).

To address how Gal-3 affected the myogenic process, we investigated the expression pattern of Gal-3 in C2C12 cells from undifferentiated myoblasts to fully differentiated myotubes. Surprisingly, the expression of Gal-3 was maintained at a relative high level until the 4th day of myogenic differentiation (D4) (Figure 3A, 3B). The expression and transcription levels of all myogenic genes were upregulated at least 30% in response to Gal-3 KD on D2 (Figure 3C). Similar changes occurred on D4, but it was Mrf4 rather than MyoD that changed markedly (Figure 3D). On D6, both primary (MyoD and MyOG) and terminal (MyHC-I and II) differentiation markers were upregulated by Gal-3 KD (Figure 3E, 3F). These exciting findings suggested an essential role of Gal-3 in regulating the myogenic ability and differentiation of C2C12 cells.

3.3 TGFβ/Smad Signaling Pathway was Upregulated in GC-induced Skeletal Muscle Loss Mice

Next, we examined the mechanisms by which Gal-3 regulates myogenesis suppression. Notably, TGFβ1 and TGFβR-I are obviously overexpressed in Dex groups, and Gal-3 KD significantly reduced their expression (Figure 4A). Co-IP results demonstrated the direct interaction between TGFβR-I and Smad3 (Figure 4B). Thus, we supposed that Gal-3 has an effect on TGFβ1 and its downstream pathway. Dex elevated the phosphorylation levels of Smad2 and Smad3, whereas Gal-3 KD restored their low-phosphorylation and inactivated state (Figure 4C, 4D). IF staining exhibited a

corresponding result (Figure 4E, 4F). These results suggested that TGFβ1-Smad2/3 signaling pathway is a critical mediator in the process of myogenic differentiation regulated by Gal-3.

3.4 Gal-3 inhibition ameliorates GC-induced muscle reduction in mice

Consistent with the *in vitro* data, TD139 partially recovered mice grip strength (Figure 5A), TA muscle mean fiber area (Figure 5B), and muscle fiber number (Figure 5C), as well as the expression levels of myogenic transcription factors (Figure 5D). Also, TD139 significantly elevated the mRNA expression levels of *Myh7*, *Myh4*, *Myh1*-encoding myosin isoforms *MyHC-I*, *MyHC-IIb*, and *MyHC-IIx* (Figure 5E). These results suggested that the inhibition of Gal-3 via TD139 had s significant improvement on Dex-induced skeletal muscle loss, which might represent a promising therapy for further clinical development.

4. Discussion

This study demonstrated that Dex-elevated levels of Gal-3 signaling induced by Dex leads to the loss of skeletal muscle mass, lean mass, and grip strength. In support of our data, impaired myoblasts' differentiation and myotubes formation function appears to be the molecular mechanism underlying the observed hypoplasia in muscles with high-expression of Gal-3. Previous studies have shown that Dex treatment decreases rat muscle regeneration capacity due to destructive changes in satellite cells [18]. Currently, there is no specific inhibitor for GCs, hampering the direct blockade of GC-induced muscle wasting from the very beginning. In this study, we found that Gal-3 is significantly upregulated in Dex-treated mice muscle, and Gal-3 KD relieved the inhibitory effects of Dex on myotube formation.

Skeletal muscle myogenesis is a multistep process that includes satellite cells generating myoblasts, followed by myoblasts proliferation and fusion and multinucleated myotube differentiation [19]. Each stage would have its own distinctive regulators to control the fate of the myoblasts. Therefore, the expression patterns of Gal-3 during the whole course of C2C12 myoblasts differentiation need to be observed. For the first time, we discovered that Gal-3 expression is at a high level for the initial 2

days and then reduces gradually during the process of myogenic differentiation in the C2C12 cell line (Figure 3A). MyoD, MyOG, Myf5 and Mrf4 are myogenic transcription factors that activate many downstream genes to initiate muscle cell differentiation, MyoD and MyOG act as marker genes at the core of muscle cell determination and early differentiation [20]. MyoD and Myf5 are generally viewed as factors involved in the determination of myogenic cells, whereas MyoG and Mrf4 are more closely associated with the terminal differentiation and homeostasis of committed myofibers [21, 22]. Therefore, the timing and expression levels of the MRFs during myogenic differentiation are tightly regulated to ensure the accurate progression of the developmental process. When Gal-3 was knocked down before the initiation of myogenic differentiation, the expression of MyoD and Mrf5 significantly increased in C2C12 myoblasts on D2; however, MyoG and Mrf4 did not show consistent changes until the 4th day of differentiation (Figure 3C, 3D). Thus, our data pointed out that myogenic differentiation is strongly affected during Gal-3 modification, which might be related to critical interactions of the regulatory effect on gene expression during myogenesis. Future studies will be performed to elucidate the mechanism underlying this inhibition. Together, our studies observed the dynamic regulation of myogenesis by Gal-3 and demonstrated its negative role in the process of skeletal muscle development.

Multiple factors and mechanisms have been shown to regulate the expression of myogenic factors and influence myogenesis. Among the pleiotropic factors that regulate skeletal muscle regeneration, TGFβ1 is one of the most studied molecules. It increases in several pathological conditions and blocks the induction of muscle-specific genes' expression and myotube formation in skeletal muscle [23, 24]. As a growth factor and cytokine belonging to a superfamily of ligands, TGFβ1 activates intracellular phosphorylation cascades by binding to the serine/threonine kinase transmembrane receptor-TGFβR [25, 26]. The canonical pathway of TGFβ1 involves TGFβR-I-mediated phosphorylation of Smad2/3 proteins which are associated with the canonical co-factor Smad4 to form tight complexes in the skeletal muscle fibers [27, 28]. This heteromeric complex of Smad2/3/4

is translocated into the nucleus, which can repress the expression of myogenic genes and some other transcriptional co-factors. On the other hand, in the non-canonical pathway, TGFβ1 regulates muscle regeneration by activating different Smads-independent pathways, such as mitogen activated protein kinases, c-Jun N-terminal kinase 1 and 2 and p38, phosphoinositide 3-kinase–protein kinase B (PKB/Akt), and nuclear factor kappa B (NF-κB) and so on [29, 30]. The molecular mechanisms underlying TGFβ1 in skeletal muscle dysfunctions are yet controversial, but evidence suggests that Smad3-mediated repression of the MyoD family is required for TGFβ1 to inhibit the terminal myogenic differentiation of myoblasts [31]. Previous studies have shown that the Smad3-mediated repression was directed at the E-box-containing muscle enhancers and the bHLH region of MyoD [32]. Interestingly, we found that reducing Gal-3 by siRNA transfection decrease the total expression levels of both TGFβ1 and TGFβR-I (Figure 4A). The direct interaction between TGFβR-I and Smad3 was proved by Co-IP in this paper (Figure. 4B). Coincidentally, Smad 2/3 activation by phosphorylation significantly increased on the 4th days of myogenic differentiation after Dex treatment, which was reversed by Gal-3 KD (Figure 4C–F). Phosphorylated Smad2/3 acted as the downstream effector of TGFβ1 to block MyoD-induced myogenic differentiation in the muscle cell nucleus, which was conformed to our results of immunofluorescence tests in C2C12 myoblasts. Thus, we speculated that Gal-3 promoted TGFβ1/Smad activation, thereby suppressing myogenic differentiation and myotube formation.

Herein, we reported that Gal-3 might exert an anti-muscular role, which opens new horizons for skeletal muscle loss therapy. It is proposed that the presence of Gal-3 drives myogenesis inhibition through the activation of TGFβ1/Smad signaling. Typically, the underlying mechanisms by which Gal-3 facilitates skeletal muscle loss are more complicated than elaborated in this study. Therefore, we cannot fully exclude other targets or signaling pathways modulated by Gal-3 in muscle loss, and a chromatin IP (ChIP)-sequencing study is warranted to identify other potential targets of Gal-3. Also,

the roles of Gal-3 in the context of skeletal muscle biology may merit a thorough investigation in follow-up studies.

5. Conclusions

In conclusion, the current data highlighted a previously unknown role for Gal-3, which shed light on how TGFβ1 coordinate with Smad2/3 signaling to regulate the expression and differentiation of myogenic transcription factors. Thus, Gal-3 mediated TGFβ1-Smad2/3 signaling in myoblasts may serve as a potential therapeutic target to preserve muscle mass.

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Ethics statement

All animal studies were approved by the research ethics committee of the School of Life Science of Fudan University (Approval No. 202101004S).

Conflict of interest/Competing interest

The authors declare that they have no competing interests.

Consent to publication

All the authors agreed to publish this article.

Data Availability Statement /Availability of Data Materials

Data from this study are available upon reasonable request to the corresponding authors.

Author Contributions

Xueying Ji, Xin Jiang, and Fan Yang designed the study and wrote the manuscript, Xueying Ji, Xin Jiang, Fan Yang, Jiaming Yu, and Mengjuan Xue performed the experiments and prepared the figures, Zhijun Bao revised the manuscript. All authors reviewed the manuscript and approved the submission.

Figure legends

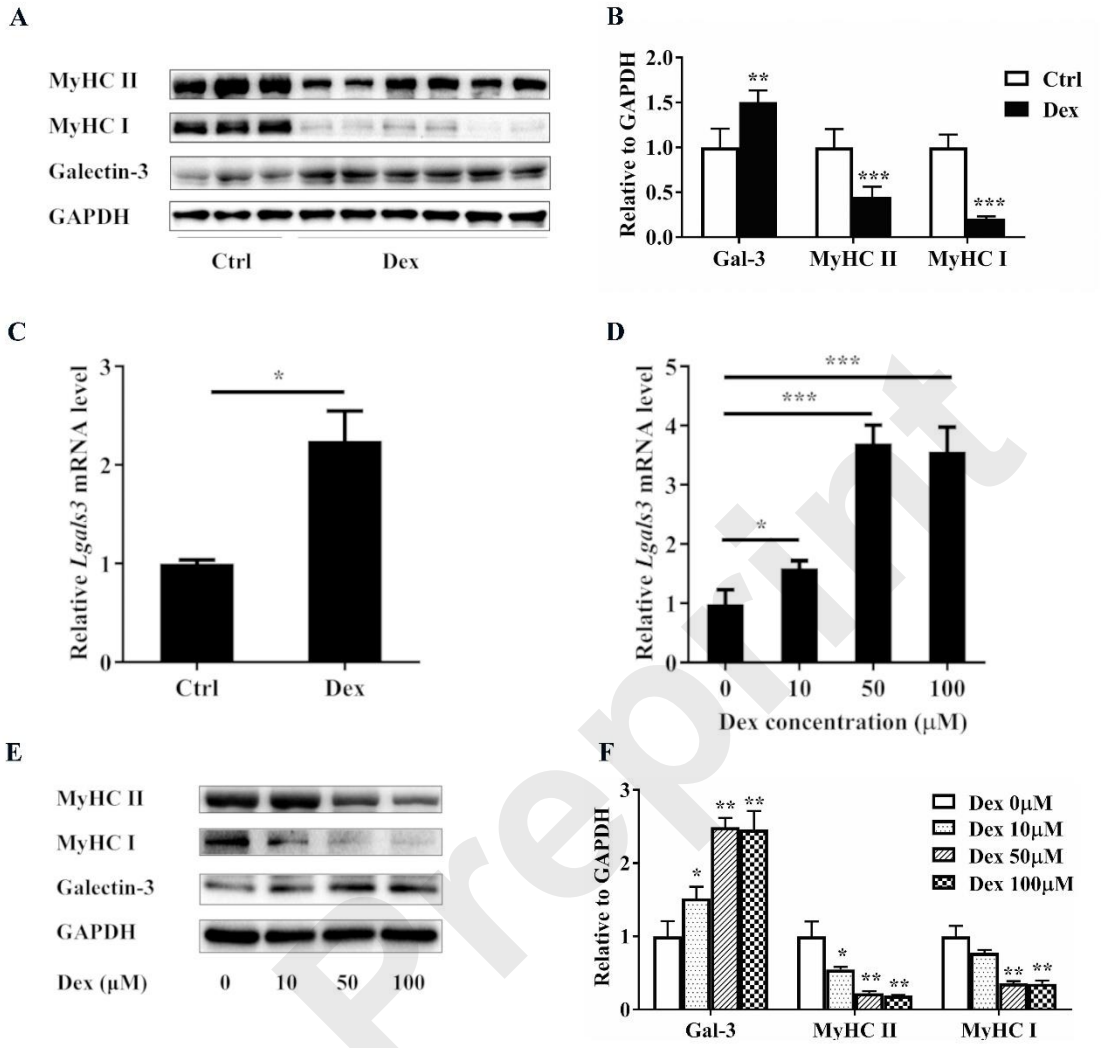


Figure 1. Gal-3 is increased in GC-induced muscle loss model. Western blotting analysis (A) and relative gray values (B) of Gal-3, MyHC-I, and MyHC-II protein levels in mice gastrocnemius muscles. (C) *Gal-3* mRNA expression levels in mice gastrocnemius muscles. (D) *Gal-3* mRNA expression levels in C2C12 myotubes treated with Dex at indicated concentrations. Western blotting analysis (E) and relative gray values (F) of Gal-3, MyHC-I, and MyHC-II protein levels in C2C12 myotubes treated with Dex at indicated concentrations. For (A–C), n=5 each group; for (D–F), n=3 each group; data are presented as mean±SD, *p<0.05, **p<0.01, and ***p<0.001.

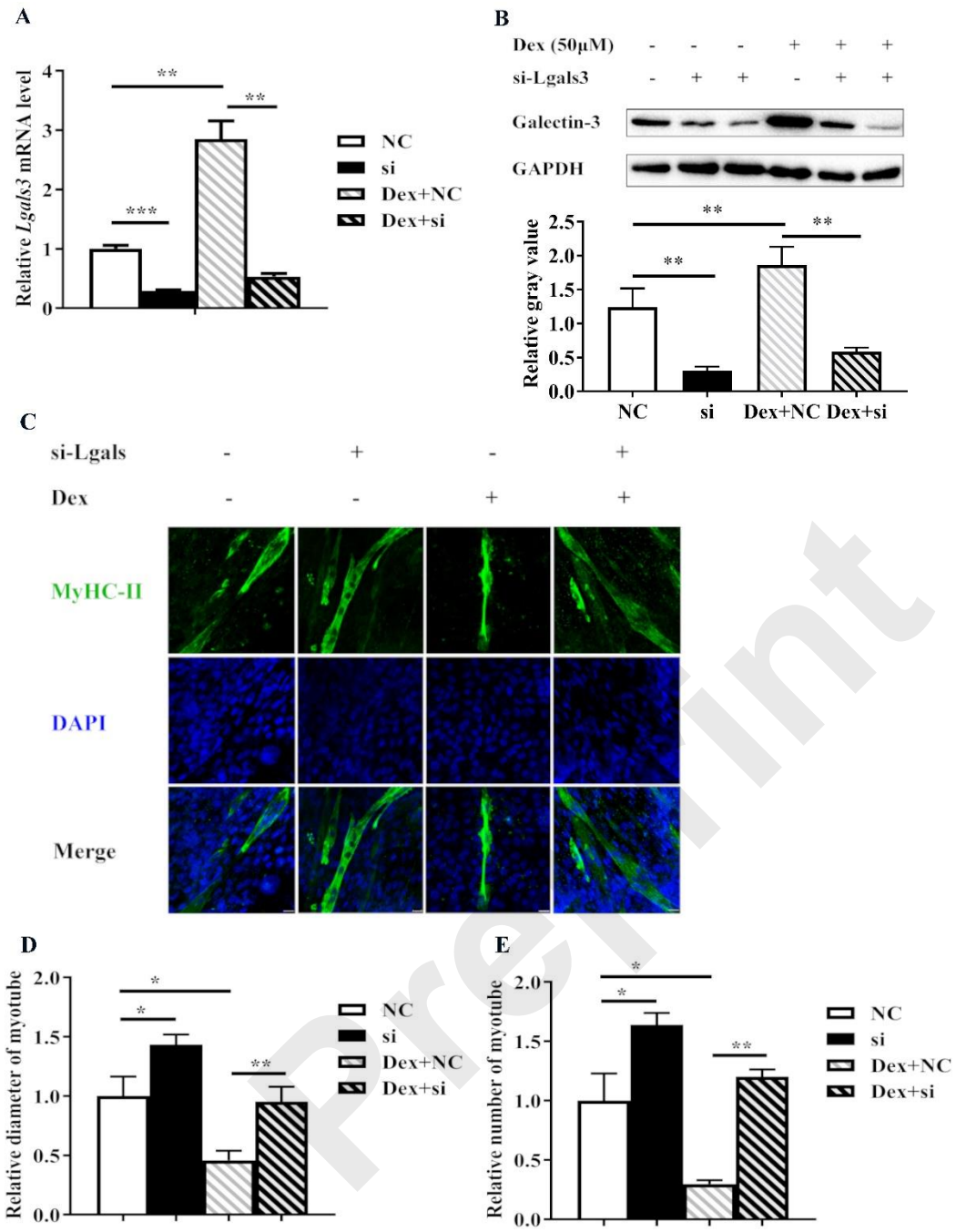


Figure 2. Gal-3 KD enlarged the size and number of myotubes *in vitro*. Interference efficiency of siRNA was measured by real-time qPCR (A) and Western blot (B). IF analysis (C) of MyHC-II was performed in C2C12 myotubes with siRNA transfection, accompanied by the relative diameter (D) and number (E) of C2C12 myotubes (scale bar: 25 μm, 10×20). Results are presented as mean±SD, n=3, *p<0.05, **p<0.01, and ***p<0.001.

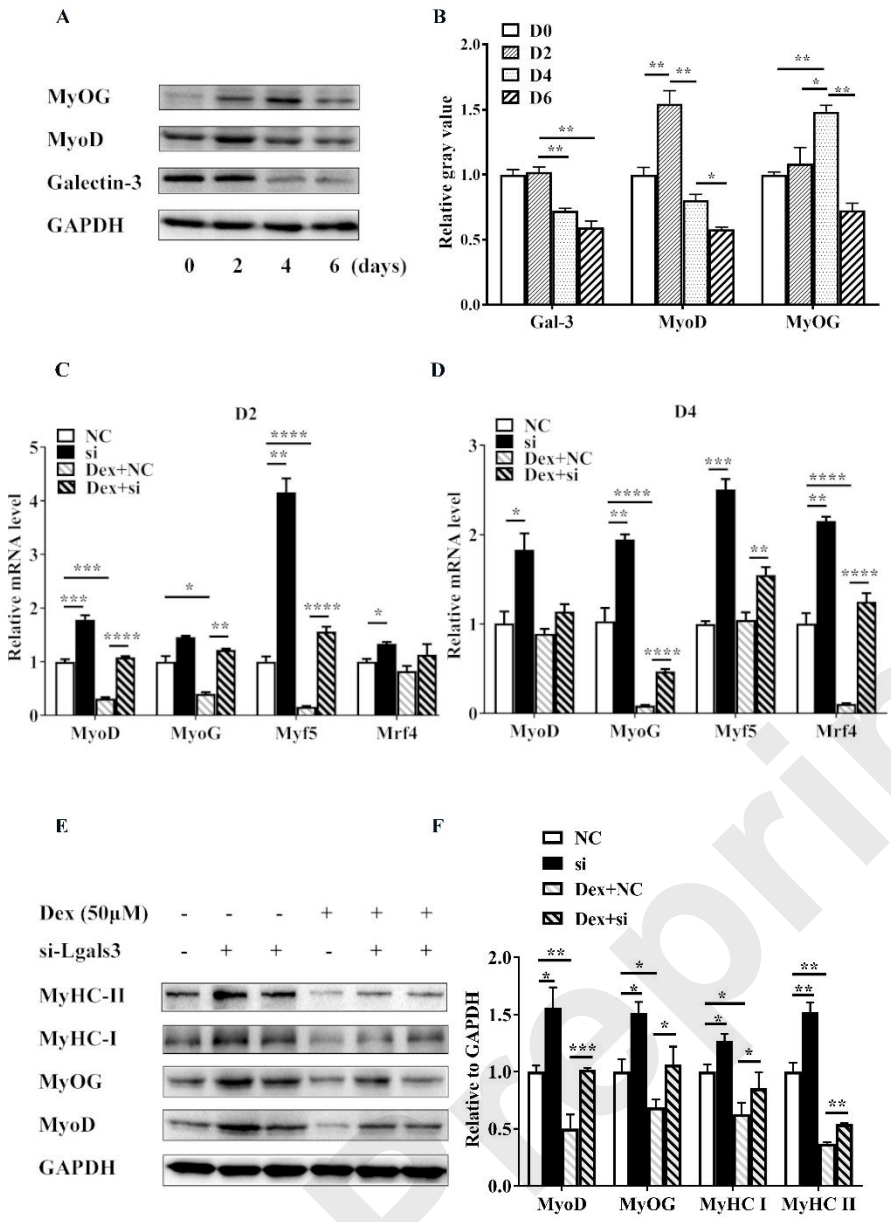


Figure 3. Gal-3 KD promoted myogenic differentiation *in vitro*. Protein levels of Gal-3, MyOG, and MyoD during C2C12 myoblast differentiation through Western blotting analysis (A) and relative gray value analysis (B). *MyoD*, *MyOG*, *Myf5*, and *Mf4* gene expression levels were analyzed by qRT-PCR on 2nd day of differentiation (C) and 4th day (D). MyHC-I, MyHC-II, MyOG, and MyoD protein levels were analyzed by Western blotting (E) and relative gray value calculation (F) on the 6th day of differentiation. Results are presented as mean±SD, n=3, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

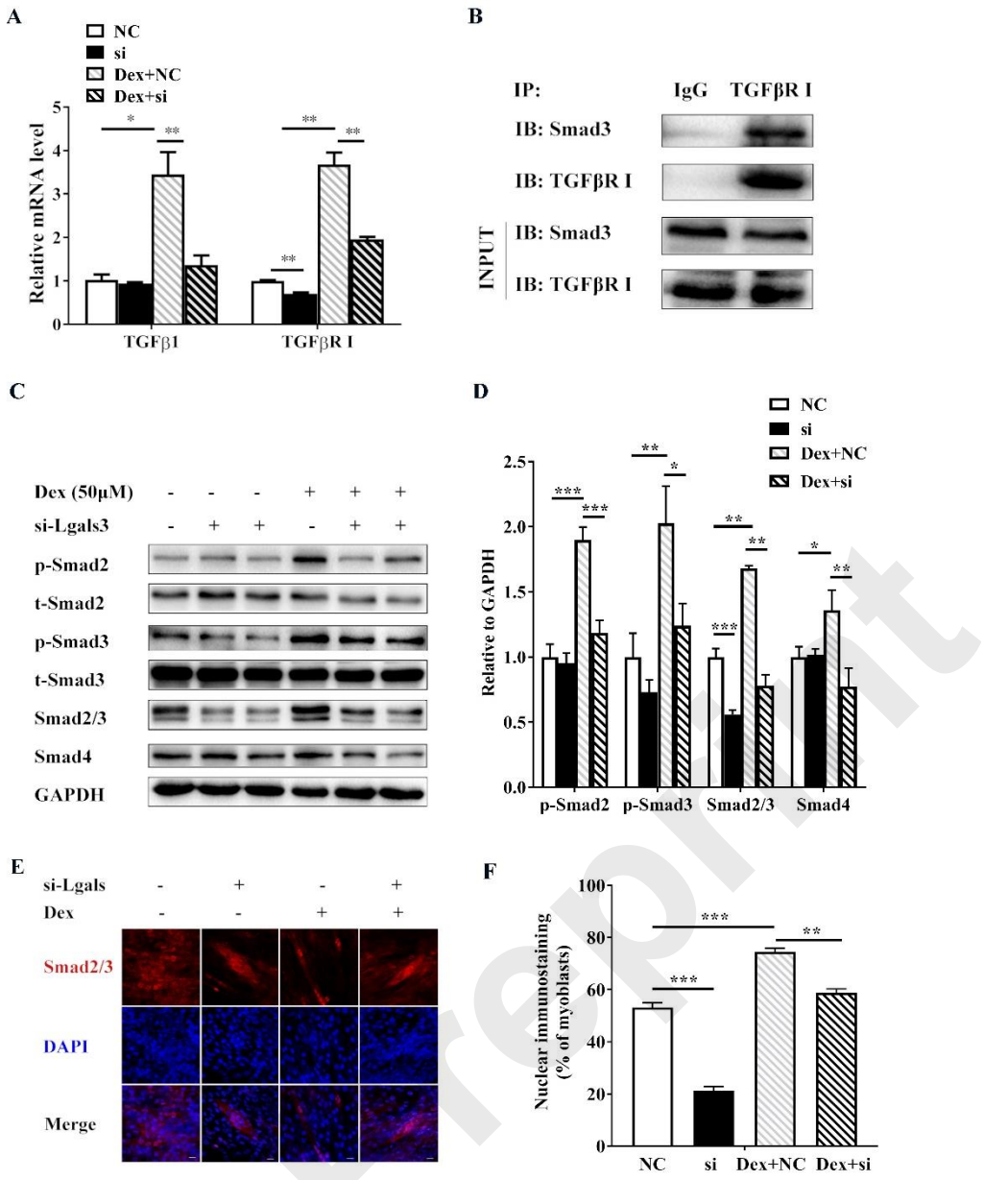


Figure 4. Gal-3 regulated Smad2/3 signaling pathway by activating TGFβ1 during myogenic differentiation. (A) The mRNA expression levels of *TGFβ1* and *TGFβR-I* are measured by qRT-PCR. (B) Co-IP validated the interaction of TGFβR-I and Smad3 in C2C12 cells. Western blotting analysis (C) and relative gray value (D) of TGFβR-I, Smad2, Smad3, and Smad4 was performed in C2C12 cells. IF analysis (E) and nuclear immunostaining ratio (F) of Smad2/3 localization in C2C12 cells (scale bar: 25 μ m, 10x20). Results are presented as means, n=3, *p<0.05, **p<0.01, and ***p<0.001.

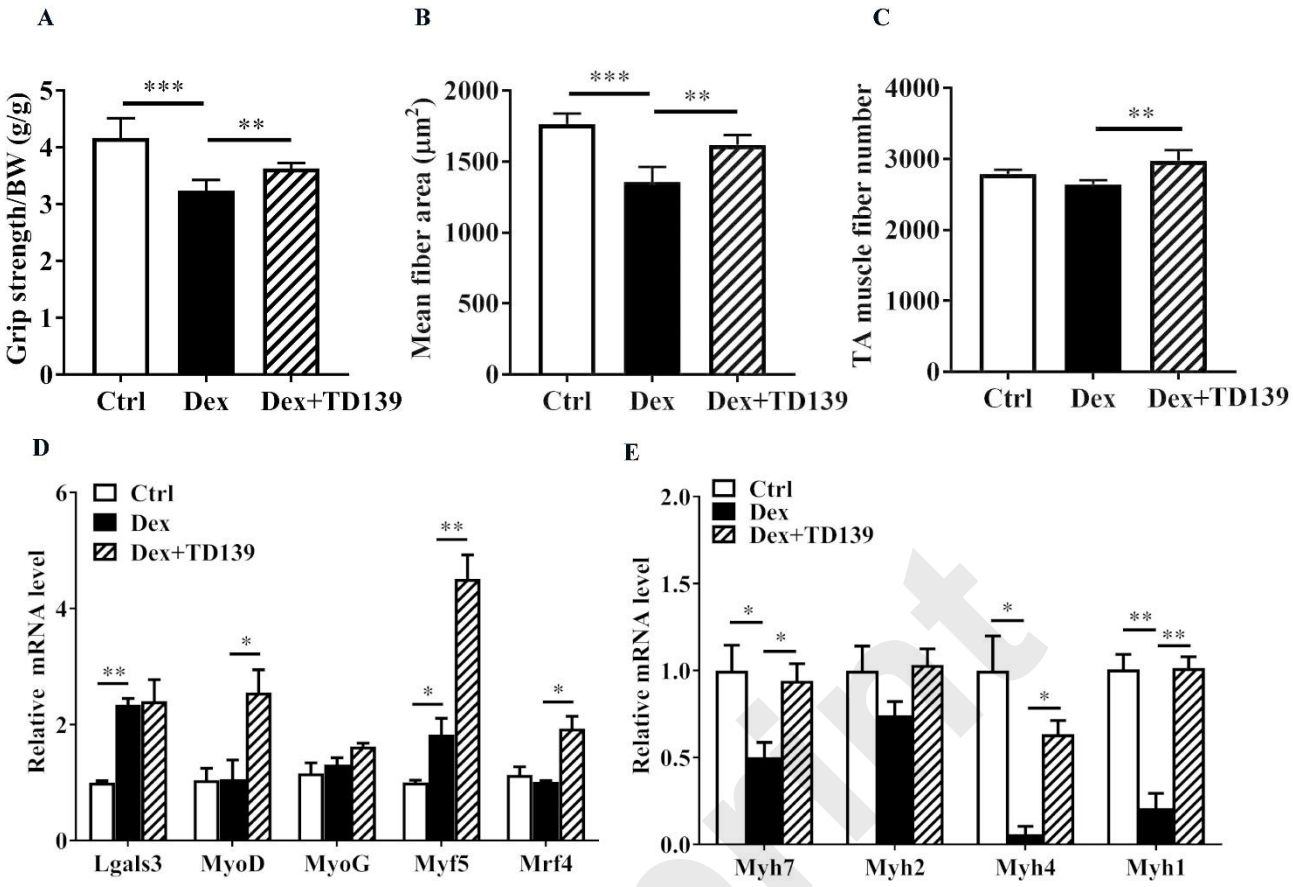
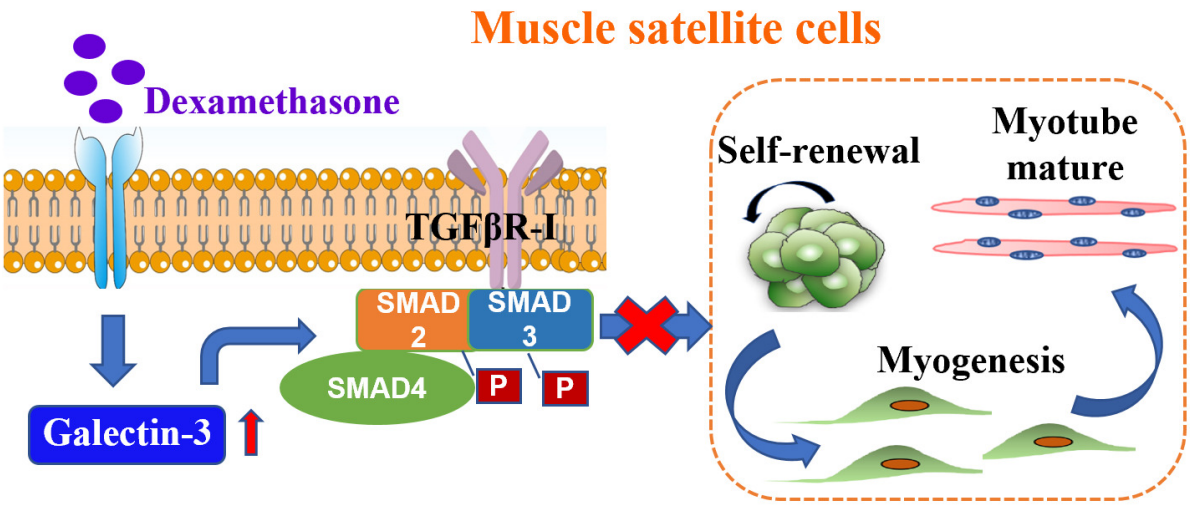


Figure 5. TD139 recovered GC-induced muscle loss in mice. (A) Grip strength, normalized to body weight (BW). (B) Mean myofiber CSA of the TA muscle. (C) TA muscle fiber number. (D) The mRNA expression levels of *Lgals3*, *MyoD*, *MyoG*, *Myf5*, and *Mrf4* in gastrocnemius muscles. (E) qRT–PCR analyzed the gene expressions of *Myh7*, *Myh2*, *Myh4*, and *Myh1* in mice gastrocnemius muscles.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TGFβR-I
SMAD 2
SMAD 3
SMAD4
P
P
Galectin-3
Self-renewal
Myotube mature
Myogenesis

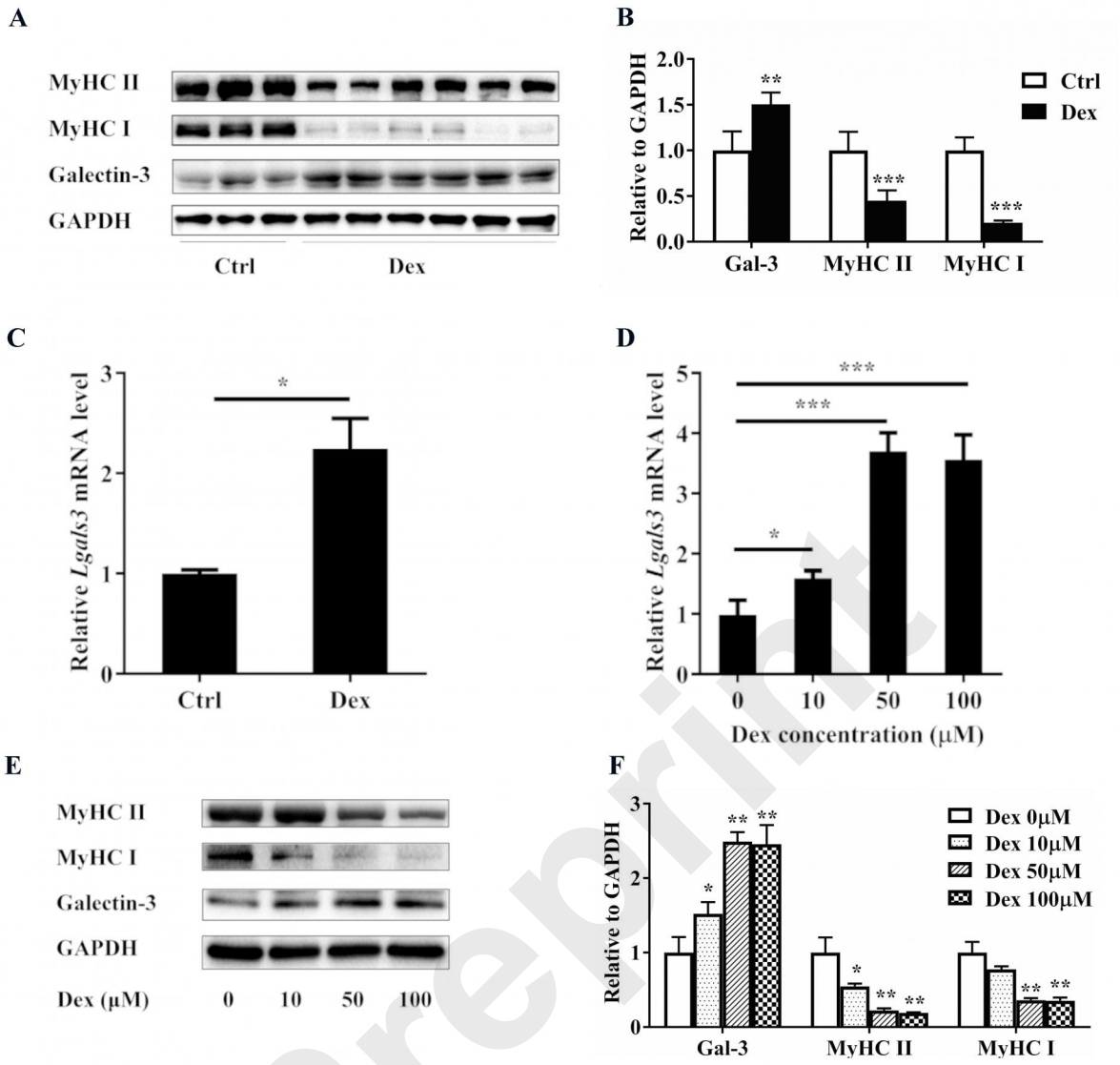


Figure 1. Gal-3 is increased in GC-induced muscle loss model. Western blotting analysis (A) and relative gray values (B) of Gal-3, MyHC-I, and MyHC-II protein levels in mice gastrocnemius muscles. (C) Gal-3 mRNA expression levels in mice gastrocnemius muscles. (D) Gal-3 mRNA expression levels in C2C12 myotubes treated with Dex at indicated concentrations. Western blotting analysis (E) and relative gray values (F) of Gal-3, MyHC-I, and MyHC-II protein levels in C2C12 myotubes treated with Dex at indicated concentrations. For (A–C), n=5 each group; for (D–F), n=3 each group; data are presented as mean±SD, *p<0.05, **p<0.01, and ***p<0.001.

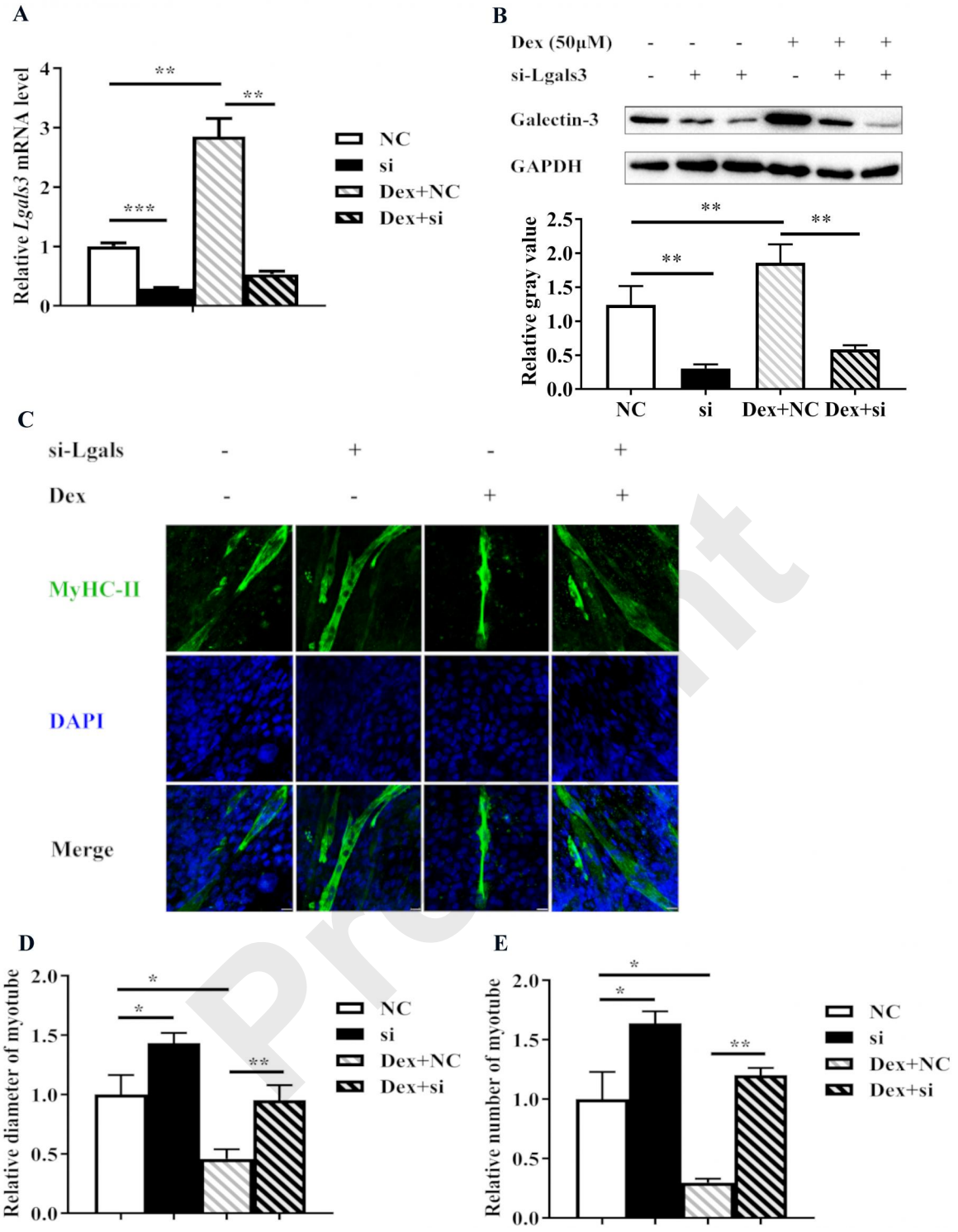
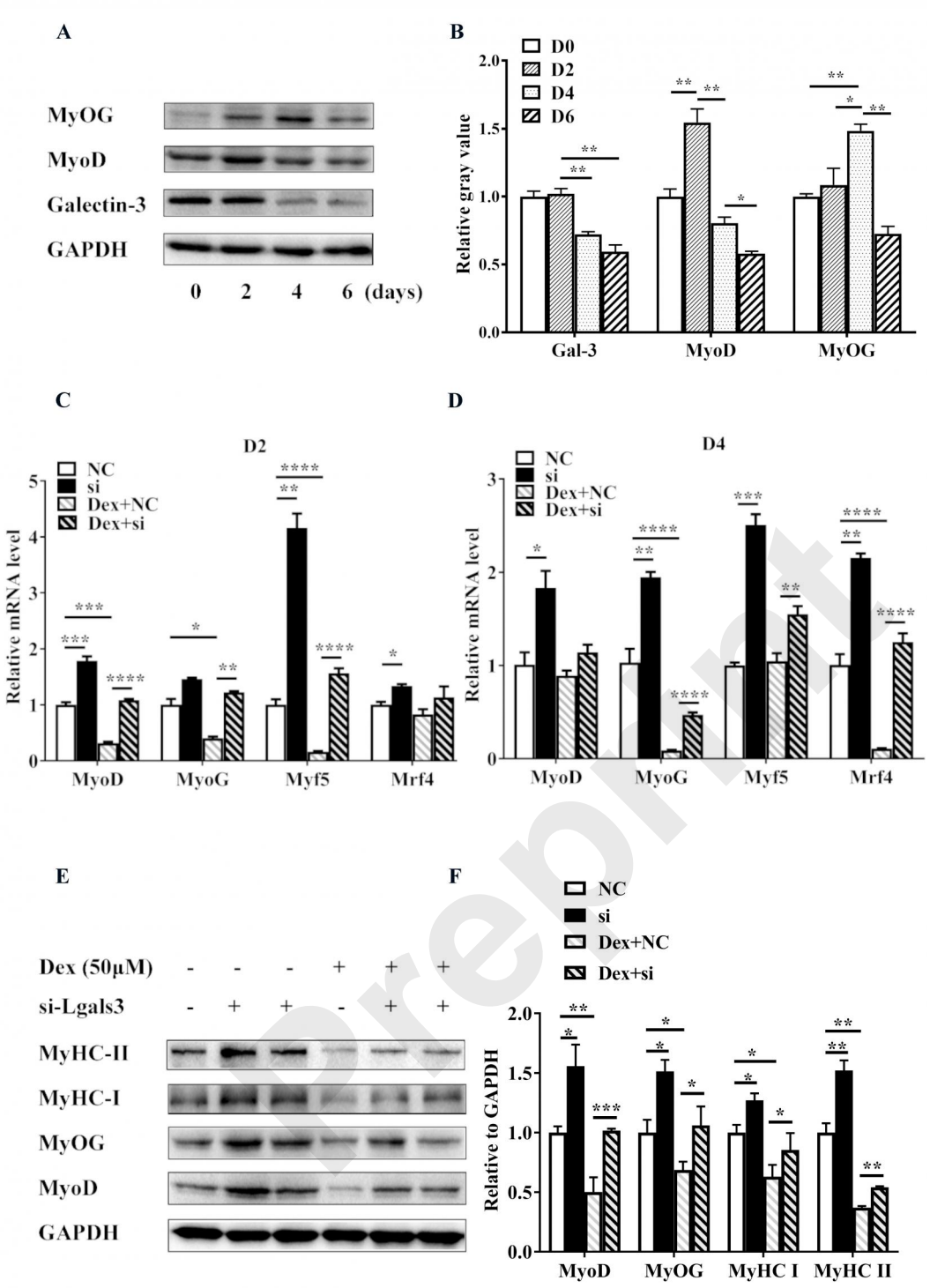


Figure 2. Gal-3 KD enlarged the size and number of myotubes in vitro. Interference efficiency of siRNA was measured by real-time qPCR (A) and Western blot (B). IF analysis (C) of MyHC-II was performed in C2C12 myotubes with siRNA transfection, accompanied by the relative diameter (D) and number (E) of C2C12 myotubes (scale bar: 25 □m, 10×20). Results are presented as mean±SD, n=3, *p<0.05, **p<0.01, and ***p<0.001.



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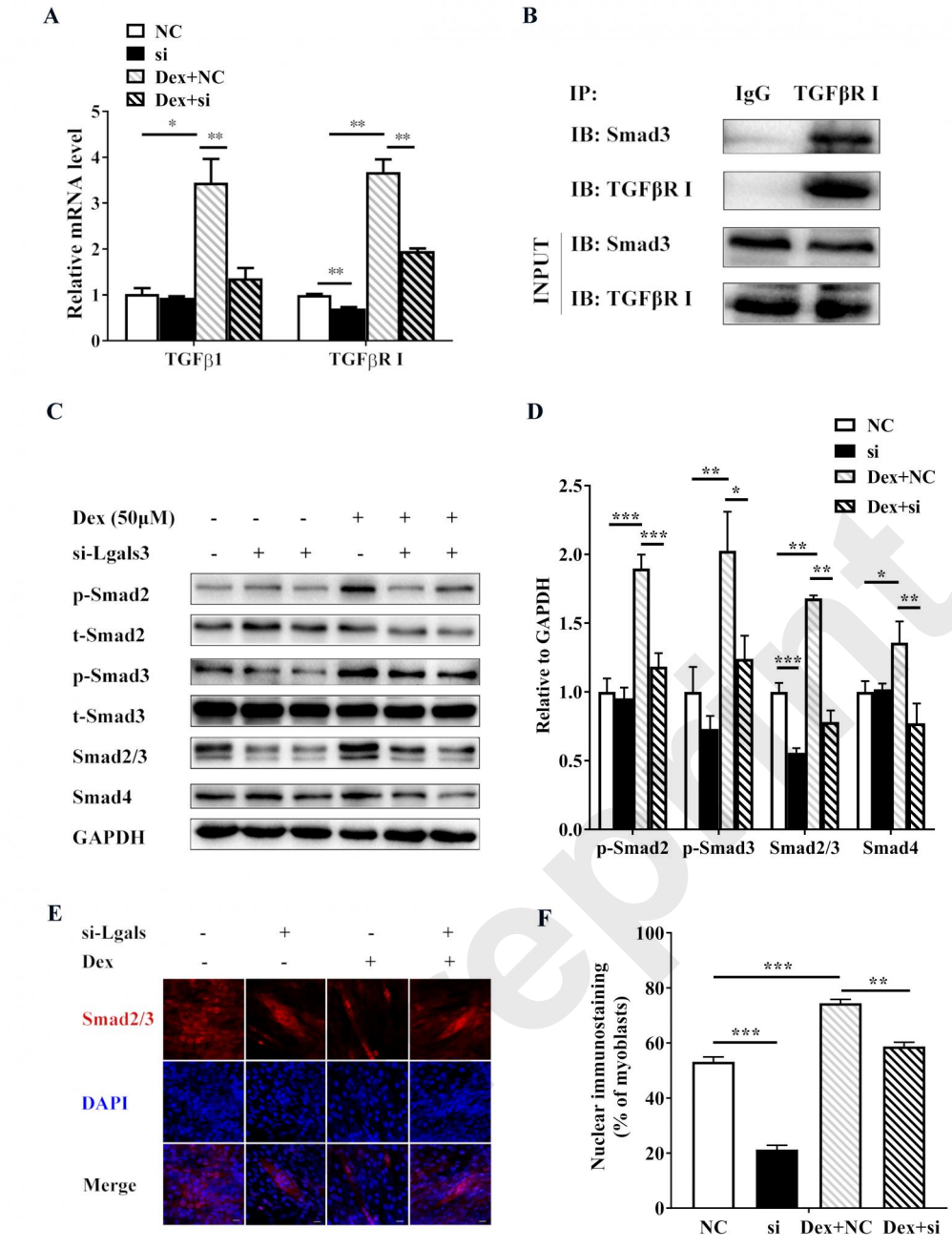

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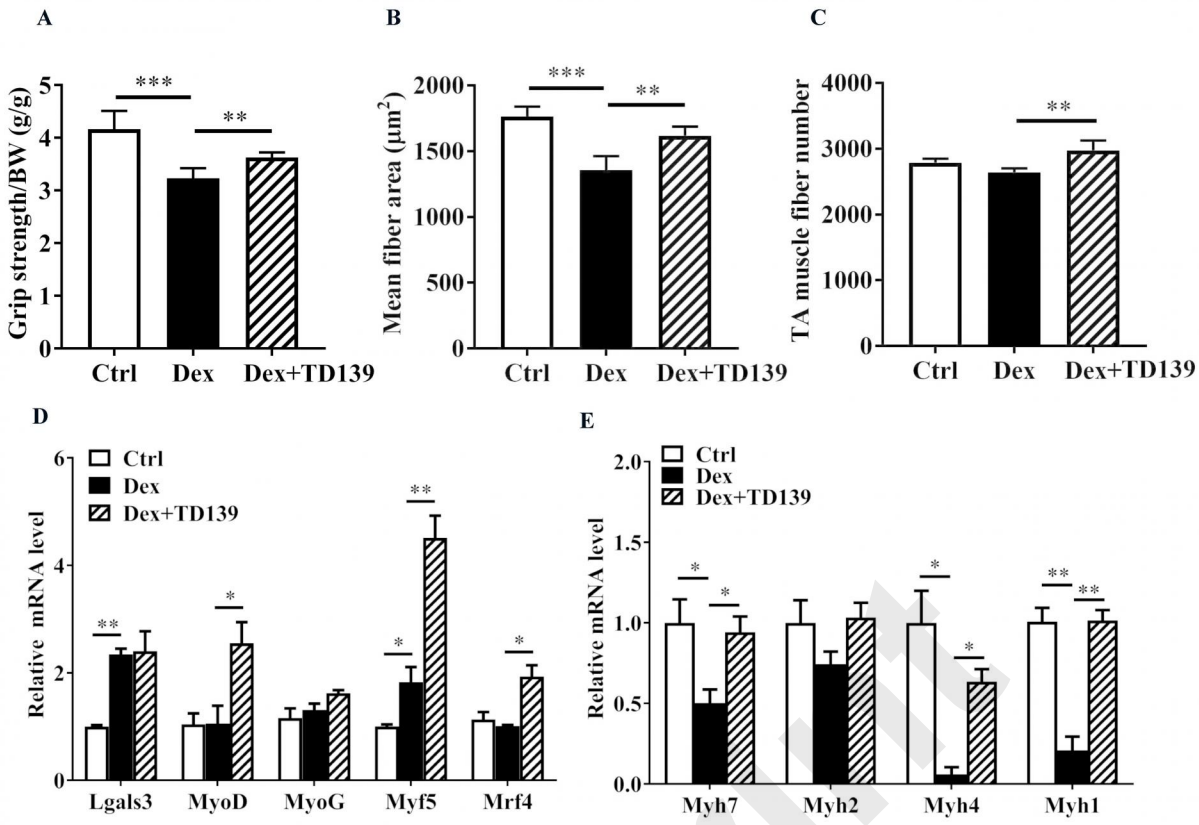

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