

p38MAPK- and Akt/mTOR-Mediated Signaling Underlies the Pro-Myogenic Effects of Ginsenoside Rg5

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ABSTRACT

Skeletal muscles are essential for both energy metabolism and physical movement. A reduction in skeletal muscle mass can lead to functional and metabolic complications. Ongoing research aims to avert these conditions by enhancing muscle mass and regenerative potential. While Ginsenoside Rg5 is known for its diverse pharmacological properties, research specifically addressing its impact on muscle development and differentiation remains limited. To explore the influence of Rg5 on myogenesis, C2C12 myoblasts underwent differentiation in the presence of Rg5. Subsequently, qRT-PCR, immunostaining, and immunoblotting were utilized to evaluate promyogenic signaling (p38MAPK) and myogenic markers. Immunoprecipitation analyses demonstrated that Rg5 enhanced the p38MAPK-mediated interaction between E2A and MyoD. To assess the protective effects of Rg5 against muscle wasting, C2C12 myotubes were exposed to dexamethasone to stimulate muscle atrophy. Molecular techniques, including qRT-PCR, immunostaining, and immunoblotting, were then applied to analyze myogenic markers, atrophy-associated genes (MuRF1 and Atrogin-1), and the Akt/mTOR protein synthesis pathway. The application of Rg5 advanced the differentiation of C2C12 myoblasts by driving MyoD/E2A heterodimerization and p38MAPK phosphorylation. Additionally, Rg5 prompted hypertrophy in C2C12 myotubes through the phosphorylation of the Akt/mTOR pathway. The resulting phosphorylation of Akt triggered FoxO3a phosphorylation, thereby downregulating the expression of both MuRF1 and Atrogin-1. This research elucidates the mechanisms by which Rg5 facilitates myogenesis and muscle hypertrophy while counteracting dexamethasone-induced atrophy. To our knowledge, this is the initial report demonstrating that Rg5 enhances muscle regeneration, highlighting its potential as a therapeutic strategy for managing muscle wasting and weakness, such as cancer cachexia.

Keywords: Ginsenoside, Glucocorticoids, Muscle atrophy, Myogenesis, Myotube growth

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Introduction

Skeletal muscles account for roughly fifty percent of total human body mass. Beyond facilitating locomotion, these tissues are critical for energy metabolism and glucose assimilation. Consequently, the depletion of skeletal muscle mass—clinically termed muscle atrophy—can result in metabolic disorders, obesity, and reduced physical mobility [1, 2].

Muscular atrophy primarily arises from impaired myoblast differentiation or a disruption in the equilibrium between protein degradation and synthesis [3-6]. Myogenesis, or skeletal muscle differentiation, is a strictly controlled, sequential mechanism. It encompasses the withdrawal from the cell cycle, the transcription of muscle-specific proteins, and the fusion of cells into multinucleated myotubes [7, 8]. Various myogenic regulatory factors, including myogenic factor 5 (Myf5) and myogenic determination gene number 1 (MyoD), orchestrate this

progression by committing cells to the myogenic pathway [9]. With advancing differentiation, there is an upregulation in the expression of specific muscle proteins, notably myosin heavy chain (MHC) and myogenin [8, 10, 11].

Multiple signaling cascades initiate myogenesis. Specifically, the p38 mitogen-activated protein kinase (p38MAPK) pathway, which is triggered by MKK6, modulates MyoD activation during muscle development. MyoD becomes active upon forming a heterodimer with E proteins that have undergone phosphorylation by p38MAPK [12]. Furthermore, p38MAPK drives myoblasts to exit the cell cycle by modulating specific chromatin remodeling enzymes and counteracting the c-Jun N-terminal kinase (JNK)/c-Jun signaling network, which typically promotes myoblast proliferation [11-14]. Research indicates that SB203580, a pharmacological agent that competitively blocks the ATP binding pocket of p38MAPK to suppress its catalytic function, disrupts the differentiation and subsequent fusion of C2C12 myoblasts [15].

Overall muscle mass relies on the balance between protein degradation and synthesis [16]. Muscle wasting occurs when the rate of protein breakdown exceeds that of synthesis. Consequently, therapeutic research has focused on stimulating protein generation while suppressing its breakdown to mitigate muscle atrophy. Within the spectrum of anabolic networks, the protein kinase B (Akt)/mammalian target of rapamycin (mTOR) cascade serves as the primary protein synthesis route essential for skeletal muscle enlargement [17]. Intracellular energy levels, amino acids, and growth factors activate this Akt/mTOR pathway, culminating in the assembly of mTORC1. Once active, mTORC1 phosphorylates eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and p70 S6 kinase 1. This action facilitates protein translation by liberating eukaryotic translation initiation factor 4E (eIF-4E) and stimulating ribosomal protein S6 [18, 19].

Prior investigations indicate that the sustained activation of Akt induces muscular hypertrophy, characterized by an expansion in the cross-sectional size of muscle fibers [20, 21]. Conversely, the depletion or deactivation of p70S6K1 and mTORC1 subunits in murine skeletal tissues precipitates a decline in muscle mass [22-24].

In opposition to anabolic pathways, proteolytic mechanisms—namely the autophagy-lysosome machinery and the ubiquitin-proteasome system (UPS)—drive muscle degradation. The activation of forkhead box O3 (FoxO3) upregulates the transcription of muscle-specific E3-ligases, including muscle atrophy F-box (MAFbx/Atrogin-1) and muscle ring finger protein-1 (MuRF1), which target proteins for breakdown [20, 25-27]. Because the phosphoinositide 3-kinase (PI3K)/Akt cascade suppresses FoxO3 activity, the stimulation of Akt signaling mitigates muscle atrophy driven by the UPS [26-28]. Therefore, the Akt/mTOR network augments muscle mass by simultaneously driving protein synthesis and restraining the proteolysis of critical sarcomeric components like actin, troponins, and MHC.

Given that skeletal muscle is central to protein and energy metabolism as well as physical mobility, numerous reports highlight its profound impact on overall quality of life and longevity [29, 30]. As a result, substantial scientific effort has been directed toward augmenting muscle regeneration and mass by boosting differentiation, elevating protein synthesis, or obstructing muscle protein degradation. For instance, research demonstrates that testosterone, which binds to androgen receptors, stimulates muscle hypertrophy mediated by Akt. Additional findings reveal that impaired differentiation can be reversed by either competitively binding to activin type II receptors (ActRIIa/b) or neutralizing myostatin, a ligand that typically impedes myoblast differentiation [31-33]. Nevertheless, despite extensive investigation, no pharmacological agent has yet cleared clinical trials, primarily due to unpredictable clinical outcomes and associated hepatotoxicity.

Ginsenoside Rg5, a bioactive constituent of ginseng, is enriched through repeated thermal processing [34, 35], making it significantly more concentrated in black ginseng compared to unprocessed ginseng. Formed via the dehydration of Rg3, Rg5 is recognized as the constitutional isomer of Rk1, a byproduct of the identical thermal procedure [36]. This compound has demonstrated a wide array of pharmacological benefits, including anticancer, anti-inflammatory, and neuroprotective properties [37-40]. Even so, research evaluating the specific impact of Rg5 on muscle growth and differentiation remains highly limited.

In the present investigation, we examined the influence of Rg5—a primary active compound in black ginseng—on the differentiation and growth of muscle tissue. Our findings indicate that Rg5 advanced myogenesis via p38MAPK phosphorylation, while simultaneously stimulating hypertrophy and averting dexamethasone-induced muscle atrophy through the Akt/mTOR signaling pathway. Collectively, these results propose that Rg5 holds promise as a viable therapeutic candidate for the management of muscle atrophy and weakness.

Materials and Methods

Cell cultivation and primary myoblast extraction

The C2C12 murine myoblast cell line was procured from the American Type Culture Collection (ATCC; No. CRL-1772, Rockville, MD, USA). These cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) enriched with 15% fetal bovine serum (FBS; Gibco) alongside an antibiotic mixture (100 units/ml penicillin and 100 µl/ml streptomycin; Welgene, Gyeongsangbuk-do, South Korea). Upon reaching 80–90% confluence, myoblast differentiation was initiated by substituting the proliferation medium with a differentiation medium consisting of high-glucose DMEM (Gibco), 2% horse serum (Gibco), and the aforementioned antibiotics (100 units/ml penicillin and 100 µl/ml streptomycin; Welgene). Additionally, the HEK293T human embryonic kidney cell line was acquired from ATCC (No. CRL-3216). These HEK293T cells were cultivated in high-glucose DMEM (Gibco) containing 10% FBS (Gibco) and the standard antibiotic formulation (100 units/ml penicillin and 100 µl/ml streptomycin; Welgene). For the purpose of immunoprecipitation assays, HEK293T cells underwent transfection using 10 µg/100pi of MyoD facilitated by Lipofectamine 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA). Subsequently, the cells were exposed to Rg5 across various growth conditions. Primary myoblasts were extracted from the hindlimb musculature of mice aged 2–3 weeks. The harvested muscle tissues were enzymatically dissociated using 1 mg/ml of a collagenase/dispase solution prepared in phosphate-buffered saline (PBS). The resulting isolated cells were propagated in a growth medium comprising 20% FBS (Gibco), 2.5 ng/ml basic fibroblast growth factor (Gibco), 10 ng/ml hepatocyte growth factor (Millipore, Billerica, MA, USA), and the requisite antibiotics (100 units/ml penicillin and 100 µl/ml streptomycin; Welgene). Differentiation in these primary cultures was prompted by exchanging the growth medium for a differentiation medium composed of high-glucose DMEM (Gibco), 2% horse serum (Gibco), and antibiotics (100 units/ml penicillin and 100 µl/ml streptomycin; Welgene). All cell cultures were incubated at 37°C in a humidified environment (95%) containing 5% CO₂.

Immunoprecipitation and western blotting

For the extraction of proteins, cellular lysis was performed utilizing a radioimmunoprecipitation assay (RIPA) buffer enriched with 10 mM sodium fluoride, 1 mM sodium vanadate, and a 1 × protease inhibitor cocktail (GenDEPOT, Katy, TX, USA). Following extraction, the samples were subjected to SDS-PAGE and subsequent western blot analysis. The specific primary antibodies utilized in this study are detailed in **Table 1**.

Table 1. List of Antibodies

Antibodies	Source	Catalog
MHC	DSHB	MF20 (hybridoma)
Myogenin	DSHB	IF05 (hybridoma)
MyoD	Santacruz	sc-32758
E2A	Santacruz	sc-133075
Phosphorylated p38	Cell signaling technology	4511
p38	Cell signaling technology	9212
Phosphorylated Akt	Cell signaling technology	4060
Akt	Cell signaling technology	9272
Phosphorylated mTOR	Cell signaling technology	5536
mTOR	Cell signaling technology	2983
Phosphorylated p70S6K	Cell signaling technology	9234
p70S6K	Santacruz	sc-2330
Atrogin-1	Santacruz	sc-166806
MuRF1	Santacruz	sc-398608
Pan-Cadherin	Santacruz	sc-59876
α-tubulin	Santacruz	sc-5286
HSP90	Santacruz	sc-13119
Alexa Fluor 488-linked anti-mouse IgG	Thermo Fisher	A28175
Alexa Fluor 555-linked anti-mouse IgG	Thermo Fisher	A28180

Western blot evaluations were also conducted on the immunoprecipitation products. Following the initial protein harvest, 1 µg of anti-E2A antibody was introduced to 1 µg of the protein lysate, and this mixture was left to incubate at 4°C for 24 h. Subsequently, Protein A agarose beads (Roche) were introduced to the solution and incubated for an additional 24 h at 4°C. The resulting immune complexes underwent three consecutive washes using RIPA buffer. Prior to western blot evaluation, the prepared samples were boiled in 2 × Laemmli sample buffer (Bio-Rad, Hercules, CA, USA).

Immunocytochemical staining

To observe the expression levels of MHC, immunostaining techniques were employed. Primary myoblasts or C2C12 cells were exposed to either the vehicle control (dimethyl sulfoxide, DMSO), Rk1, or Rg5. Following treatment, cellular fixation was achieved using 4% paraformaldehyde. The cells were then permeabilized utilizing 0.5% Triton X-100 diluted in PBS, subjected to a blocking step, and subsequently incubated with an anti-MHC antibody at 4°C for 24 h. Afterward, the samples were treated with a fluorescently labeled secondary antibody (Alexa Fluor 594), and visual data were captured employing an Axio Observer Z1 LSM 700 microscope (Zeiss).

Assessment of cell viability

Cellular viability was assessed utilizing the MTT assay. C2C12 myoblasts were plated into 96-well culture dishes and given a 24 h incubation period to facilitate adherence to the substrate. Various ascending concentrations of Rg5 were subsequently introduced, followed by an additional 24 h of incubation. Each well then received 0.1 mg of MTT, and the plates were incubated for 3 h to enable the reduction of the tetrazolium dye. The resulting formazan crystals were dissolved by adding 200 µl of DMSO, and the optical density was quantified at a wavelength of 560 nm. Background absorbance readings obtained at 670 nm were subtracted from the final measurements.

RNA isolation and quantitative RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was employed to isolate total RNA from the C2C12 cultures. The extracted RNA was reverse-transcribed into cDNA utilizing iScript Reverse Transcriptase (Bio-Rad). Subsequent qRT-PCR evaluations were executed utilizing a 2 × SYBR Green mix (MGmed, Seoul, South Korea). The expression levels of target genes were standardized against the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specific primer sequences implemented during this procedure are provided in **Table 2**.

Table 2. Compilation of Primer Sequences

Gene	Sequences	
	Forward	Reverse
MyoG	ATC TCC GCT ACA GAG GCG GG	TAG GGT CAG CCG CGA GCA AA
MyHC7	CCA TCT CTG ACA ACG CCT ATC	GGA TGA CCC TCT TAG TGT TGA C
MyHC2	AAG TGA CTG TGA AAA CAG AAG CA	GCA GCC ATT TGT AAG GGT TGA C
MyHC1	TTC ATT AGT TTC CCA GCT CTC C	AGC CAC TCT TGG CCT TTA TC
MyHC4	GAT TGA CGT GGA GAG GTC TAA C	CCT GAG TTT CCT CGT ACT TCT G
Atrogin-1	CAT GCG GTT CAA GCC ACT G	GTC TGG TTA CAC TTG GTG GAG
MuRF1	GAG TGG GTT TGG AGA CAA AGA	CCA GCA TGG AGA TGC AGT TA

Statistical evaluation

The numerical data and values presented within the Figure captions are expressed as the mean ± standard deviation (SD) or the standard error of the mean (SEM). To evaluate statistical significance between two distinct groups, an unpaired two-tailed Student's t-test was utilized. When comparing multiple experimental groups, statistical significance was ascertained via one-way or two-way analysis of variance (ANOVA). Thresholds for statistical significance were established at *P < 0.05, **P < 0.01, and ***P < 0.001.

Results and Discussion

Ginsenoside Rg5, unlike Rk1, stimulates myogenesis

Prior literature demonstrates that black ginseng bolsters the myogenic process [41–43]. Within the diverse constituents of black ginseng, the concentrations of both Rk1 and Rg5 are elevated as a result of cyclical drying and steaming procedures. Consequently, we postulated that the pro-myogenic properties observed in black ginseng are attributable to the activity of either Rk1 or Rg5 (**Figure 1a**). To determine the individual impacts of these compounds, C2C12 myoblasts were prompted to undergo differentiation in the presence of 100 nM of either Rk1 or Rg5. Following this treatment, we evaluated the protein expression levels of myogenin and MHC. While C2C12 cells treated with Rg5 exhibited elevated expression of both myogenin and MHC, cultures treated with Rk1 displayed no significant variance when compared to the vehicle-treated control group (**Figure 1b**).

Immunostaining assessments for MHC revealed a higher prevalence of multinucleated cells within the Rg5-treated cohorts relative to those exposed to the vehicle (DMSO). In contrast, the Rk1-treated groups demonstrated no such notable alterations (**Figures 1c and 1d**). Prompted by these findings, we investigated the phosphorylation status of p38MAPK, a key signaling mediator in muscle differentiation. We noted enhanced p38MAPK phosphorylation in the cells subjected to Rg5, whereas the Rk1-treated samples lacked any significant modulation (**Figure 1e**). Ultimately, these outcomes indicate that Rg5 possesses myogenic stimulatory effects, which are likely linked to the induction of p38MAPK phosphorylation.

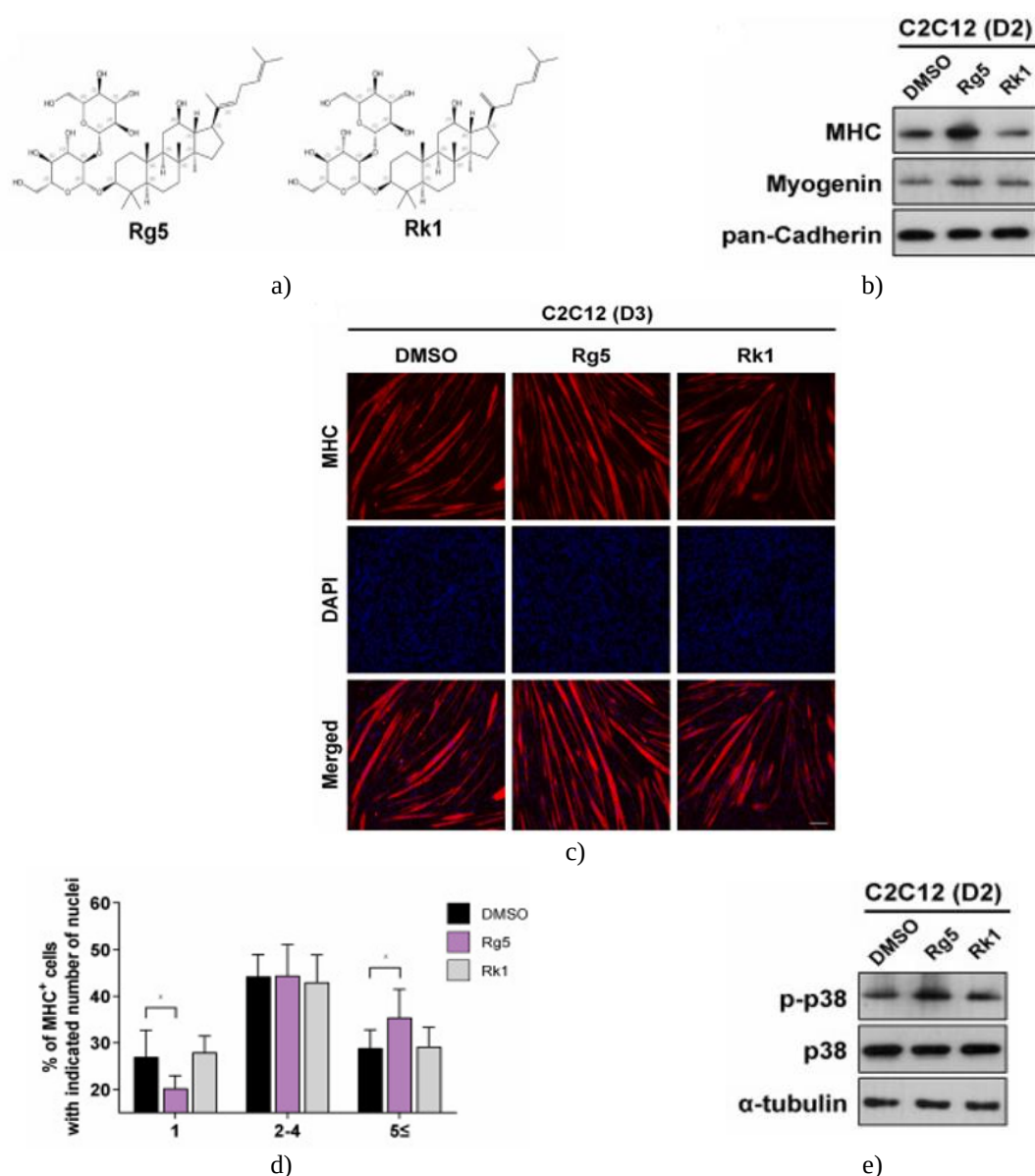
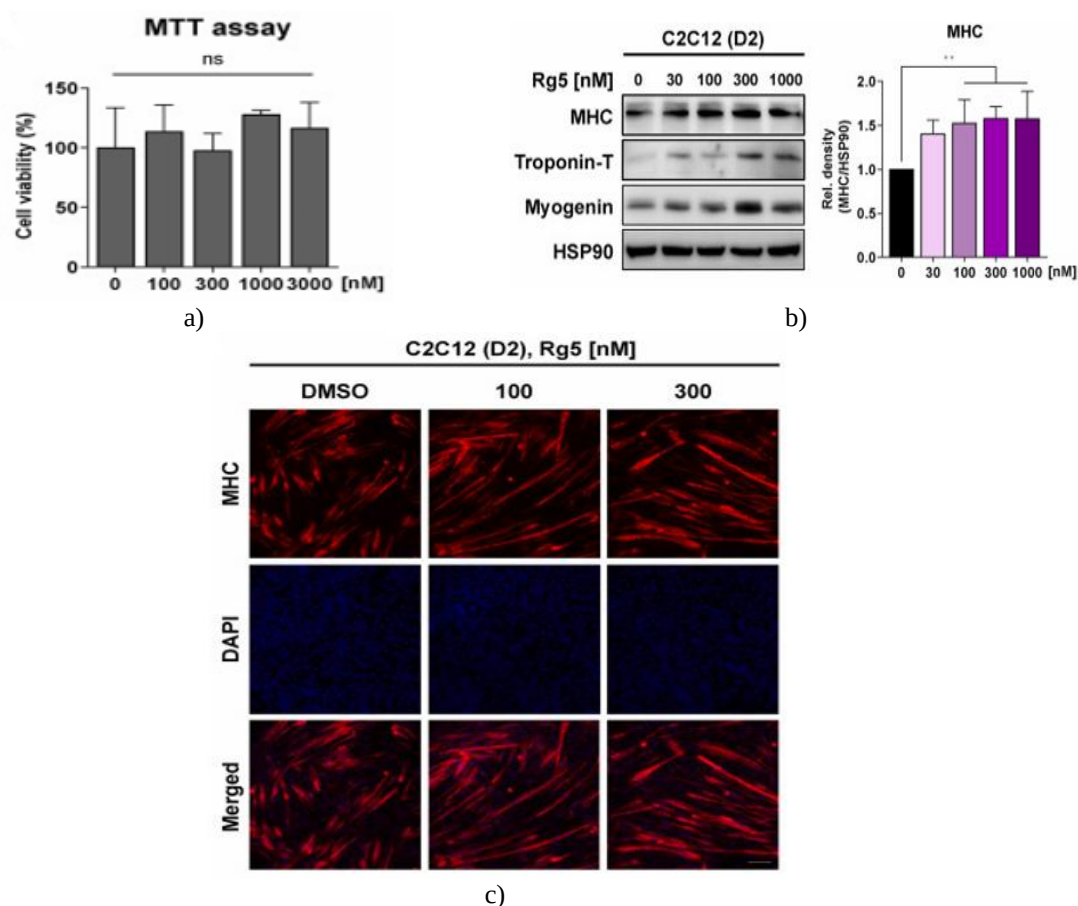


Figure 1. Ginsenoside Rg5, unlike Rk1, facilitates muscle differentiation. (a) Chemical conformations of Rk1 and Rg5. (b) C2C12 myoblasts were stimulated to differentiate for 48 h (D2) within a differentiation medium

containing either 100 nM Rk1, 100 nM Rg5, or a vehicle control (DMSO). Western blot techniques were employed to assess the protein levels of myogenin and MHC, which serve as myogenic indicators. (c) C2C12 cultures were subjected to a 72 h (D3) differentiation period in medium supplemented with 100 nM Rk1, 100 nM Rg5, or the vehicle (DMSO), and subsequently immunostained to detect MHC. Nuclear structures were highlighted via DAPI staining (blue). Scale bar = 100 μ m. (d) Quantitative analysis of the immunostained C2C12 myotubes presented in **Figure 1c**. The provided values denote the fraction of MHC-positive cells exhibiting the specified nuclear counts, determined by dividing the quantity of multinucleated MHC-positive cells by the overall count of MHC-positive cells ($N = 4$). Data are expressed as the mean $\pm$ SD. Statistical relevance was evaluated utilizing a two-way ANOVA. * $P < 0.05$; Rg5 compared to the vehicle (DMSO). (e) C2C12 cells cultivated for 48 h (D2) in differentiation medium infused with 100 nM Rk1, 100 nM Rg5, or the vehicle (DMSO). The abundance of total p38MAPK and phosphorylated p38MAPK was scrutinized through western blotting, with pan-cadherin functioning as the internal loading control.

Ginsenoside Rg5 enhances myogenesis in a dose-dependent fashion

Following the verification of Rg5's myogenic properties, a sequence of assays was performed to evaluate whether this response acts dose-dependently and to identify the optimal concentration for maximizing myogenic marker expression. Initially, the impact of Rg5 on C2C12 cell viability was assessed. Viability remained largely unaffected at Rg5 concentrations ranging from 0 to 3 μ M (**Figure 2a**). Subsequently, C2C12 cultures were exposed to distinct Rg5 concentrations. A dose-dependent elevation in the expression of myogenin, troponin-T, and MHC was observed up to an Rg5 concentration of 300 nM. At 1 μ M, however, the myogenic response did not increase proportionally (**Figure 2b**). Consequently, 300 nM was established as the ideal concentration, representing the lowest dose capable of inducing the maximum effect. Furthermore, the formation of multinucleated myotubes possessing ≥ 5 nuclei exhibited a significant, dose-dependent rise following Rg5 administration (**Figures 2c and 2d**).



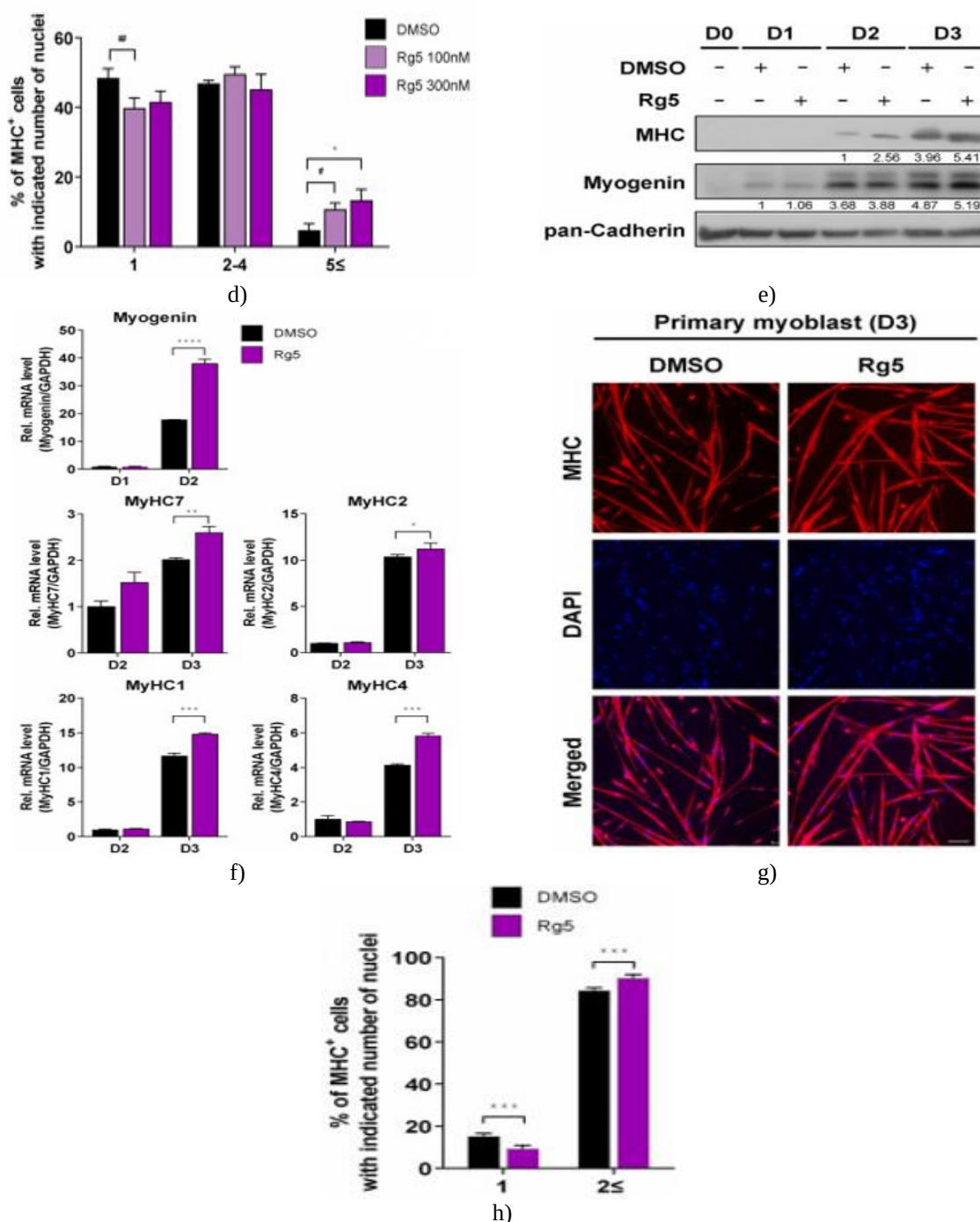


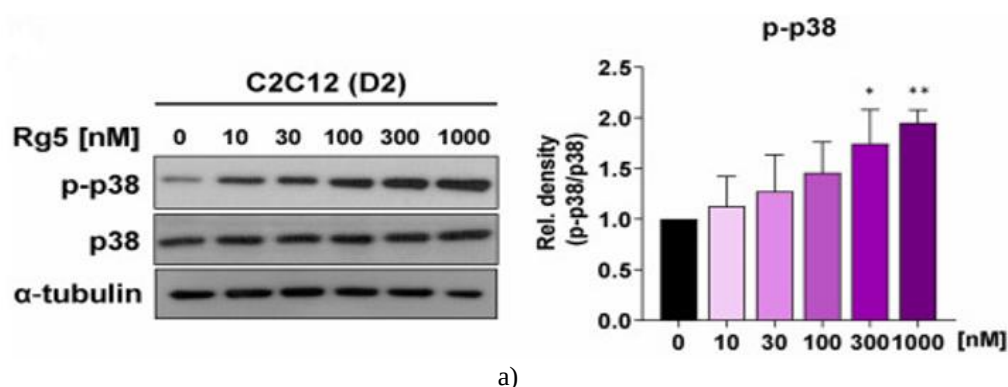
Figure 2. Ginsenoside Rg5 stimulates myogenic differentiation in a dose-dependent fashion. (a) Findings from the MTT assay for C2C12 cells exposed to either the vehicle (DMSO) or Rg5 (100, 300, 1000, or 3000 nM). Cellular viability is presented as the ratio of the 560 nm/670 nm absorbance relative to the vehicle control. (b) C2C12 cultures grown in differentiation medium for 48 h (D2) in the presence of vehicle (DMSO) or Rg5 (30, 100, 300, or 1000 nM). Western blot analysis was utilized to evaluate the expression of the myogenic markers myogenin, troponin-T, and MHC. MHC levels were standardized against HSP90 and are displayed as fold changes relative to the DMSO control. Data indicate the mean $\pm$ SEM of the fold changes across three distinct experiments. One-way ANOVA was applied to assess statistical significance. * $P < 0.05$, ** $P < 0.01$; Rg5 compared to vehicle (DMSO). (c) C2C12 myotubes cultivated for 48 h (D2) in differentiation media containing vehicle (DMSO) or Rg5 (100 or 300 nM) were subsequently immunostained to detect MHC. DAPI staining was used to illuminate the nuclei (blue). Scale bar = 100 μ m. (d) Quantitative analysis of the immunostained C2C12 myotubes presented in **Figure 2c**. The data illustrate the fraction of MHC-positive cells possessing the specified nuclear count, determined by dividing the quantity of

multinucleated MHC-positive cells by the overall number of MHC-positive cells, inclusive of mononucleated ones ($N = 4$). Results are expressed as the mean $\pm$ SD. Two-way ANOVA was utilized to calculate statistical significance. * $P < 0.05$; 300 nM Rg5 compared to vehicle (DMSO). ## $P < 0.01$, # $P < 0.05$; 100 nM Rg5 compared to vehicle (DMSO). (e) C2C12 cells cultured over the designated time points (D0–D3) in the presence of vehicle (DMSO) or 300 nM Rg5. Western blotting was performed to detect the expression of myogenin and MHC, with pan-Cadherin serving as the loading control. Expression levels for both myogenic markers were standardized to pan-Cadherin and are reported as fold changes relative to the DMSO vehicle. (f) C2C12 cultures exposed to vehicle (DMSO) or 300 nM Rg5 for 24 h (D1), 48 h (D2), and 72 h (D3). The relative mRNA levels for myogenin are shown as fold changes compared to the 24 h (D1) vehicle (DMSO) group. The relative mRNA levels for MyHC 7, 2, 1, and 4 are provided as fold changes relative to the 48 h (D2) vehicle (DMSO) group. All transcript levels were standardized against GAPDH within the respective samples. Results are shown as the mean $\pm$ SEM. Statistical differences were evaluated via one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Rg5 relative to vehicle (DMSO). (g) Primary myoblasts grown for 72 h (D3) in differentiation media containing vehicle (DMSO) or 300 nM Rg5 were subjected to MHC immunostaining. DAPI was employed to highlight the nuclei (blue). Scale bar = 100 μ m. (h) Quantitative assessment of the immunostained primary myoblasts displayed in **Figure 2g**. Results denote the percentage of MHC-expressing cells harboring the specified nuclear count, computed by dividing the number of multinucleated MHC-positive cells by the total pool of MHC-positive cells, encompassing mononucleated ones ($N = 5$). Values are given as the mean $\pm$ SD. Statistical differences were assessed using two-way ANOVA. *** $P < 0.001$; Rg5 relative to vehicle (DMSO).

Next, we explored whether Rg5's influence persisted throughout the entire differentiation period. From the second to the third day, cells treated with Rg5 exhibited elevated expression of myogenin and MHC relative to the control subjects. Furthermore, Rg5 administration elevated myogenin mRNA levels, which subsequently upregulated the expression of multiple MyHC gene isotypes responsible for encoding distinct MHC variants (**Figures 2e and 2f**). Moreover, the myogenic impact of Rg5 was evaluated in primary myoblasts, providing an effective *ex vivo* model for investigating muscle tissues. Primary myoblasts were harvested from the hindlimb musculature of 2–3-week-old mice, and differentiation was stimulated through Rg5 application. Findings indicated a higher quantity of fused myoblasts in the Rg5-treated cultures relative to the controls (**Figures 2g and 2h**). Collectively, these data demonstrate that Rg5 exerts concentration-dependent myogenic benefits, a phenomenon apparent in primary myoblasts as well as C2C12 cell lines.

Rg5 drives myogenesis via p38MAPK and Akt phosphorylation

The process of myogenesis is driven by p38MAPK phosphorylation and the ensuing stimulation of transcription factors [12–14]. We explored if Rg5's myogenic influence relies on p38MAPK phosphorylation. Rg5 induced a progressive, dose-dependent elevation in p38MAPK phosphorylation levels (**Figure 3a**). Furthermore, to ascertain if blocking p38MAPK would suppress the myogenic benefits of Rg5, cultures were exposed to both Rg5 and a p38MAPK inhibitor (SB203580). While Rg5 administration alone enhanced myogenin and MHC expression, the introduction of the inhibitor reduced these expression levels, even when Rg5 was present. Such findings suggest that the p38MAPK signaling cascade is linked to the myogenic properties of Rg5 (**Figure 3b**).



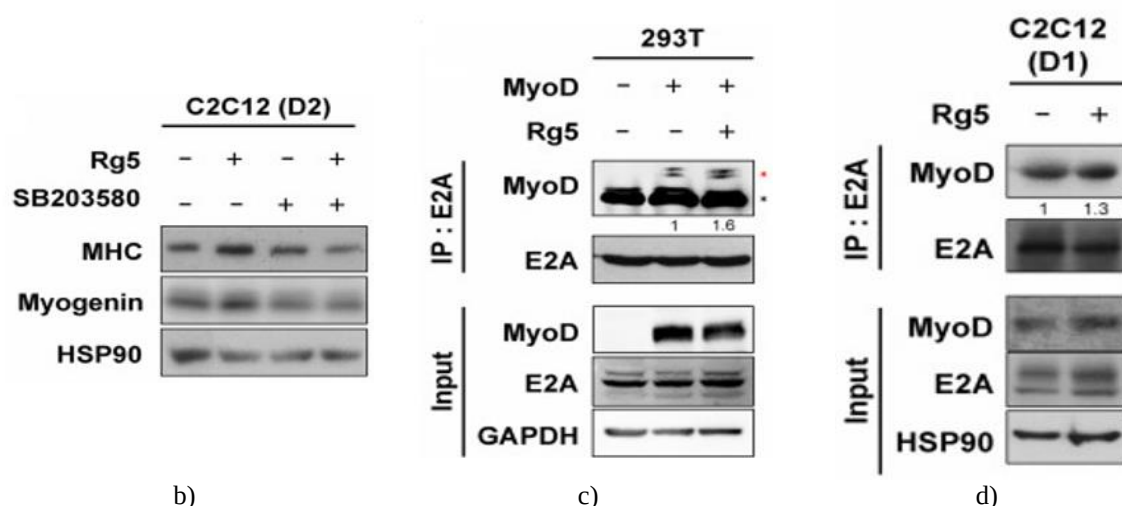


Figure 3. Rg5 facilitates myogenesis through the phosphorylation of p38MAPK and Akt. (a) C2C12 cultures cultivated for 48 h (D2) in differentiation media containing vehicle (DMSO) or Rg5 (30, 100, 300, or 1000 nM). Western blotting was utilized to assess the degree of p38MAPK phosphorylation. Phosphorylated p38MAPK levels were standardized against total p38MAPK and are presented as fold changes relative to the vehicle (DMSO) control. Data represent the mean $\pm$ SEM of the fold changes obtained across three separate trials. One-way ANOVA was utilized to evaluate statistical significance. * $P < 0.05$, ** $P < 0.01$; Rg5 compared to vehicle (DMSO). (b) C2C12 cultures sustained for 48 h (D2) in differentiation media supplemented with vehicle (DMSO), SB203580 (100 nM), or Rg5 (300 nM). Western blot analysis was performed to measure the expression of the myogenic markers myogenin and MHC, utilizing HSP90 as the loading control. (c) HEK293T cells transfected with MyoD were exposed to vehicle (DMSO) or Rg5 (300 nM) for a 24-h period. Cellular lysates underwent immunoprecipitation using E2A antibodies, followed by immunoblotting with E2A and MyoD antibodies. Red stars denote MyoD bands and black stars indicate IgG bands. The input serves as a control for expression. The binding affinity between E2A and MyoD following Rg5 application was quantified and is displayed as a fold change compared to the DMSO-treated vehicle control. (d) C2C12 cultures grown in differentiation media containing vehicle (DMSO) or Rg5 (300 nM) for 24 h (D1). Lysates from the cells were immunoprecipitated utilizing E2A antibodies and subsequently immunoblotted with MyoD and E2A antibodies. The input acts as an expression control. The resulting binding affinity between E2A and MyoD under Rg5 treatment was measured and is presented as a fold change relative to the vehicle (DMSO) control.

As previously noted, p38MAPK phosphorylation stimulates myogenesis by enhancing the association between MyoD and E2A [12]. To determine if Rg5-mediated p38MAPK phosphorylation amplifies this MyoD-E2A interaction, immunoprecipitation assays were conducted. Following Rg5 administration, a heightened interaction was detected in both C2C12 cultures and MyoD-transfected HEK293T cells (**Figures 3c and 3d**). Consequently, these findings indicate that the myogenic influence of Rg5 is linked to elevated p38MAPK phosphorylation and augmented MyoD activity.

Ginsenoside Rg5 induces muscle hypertrophy via Akt phosphorylation and mitigates dexamethasone-triggered atrophy

Drawing upon prior research illustrating that black ginseng promotes muscle hypertrophy [41-43], the capacity of Rg5 to encourage hypertrophic growth and avert muscle wasting was investigated. Rg5 and dexamethasone were administered to C2C12 myotubes. MHC expression was upregulated following Rg5 application but diminished upon dexamethasone exposure. Nevertheless, concurrent application of dexamethasone and Rg5 recovered MHC expression levels (**Figure 4a**). Furthermore, to establish if Rg5 could drive hypertrophy—evidenced by enlarged myotube diameters—and counteract dexamethasone-stimulated atrophy, MHC immunostaining was conducted under identical experimental conditions. Myotube diameter expanded in the Rg5-treated group, whereas it contracted in the dexamethasone-treated group. Consistent with expectations, simultaneous treatment rescued myotube thickness (**Figures 4b and 4c**).

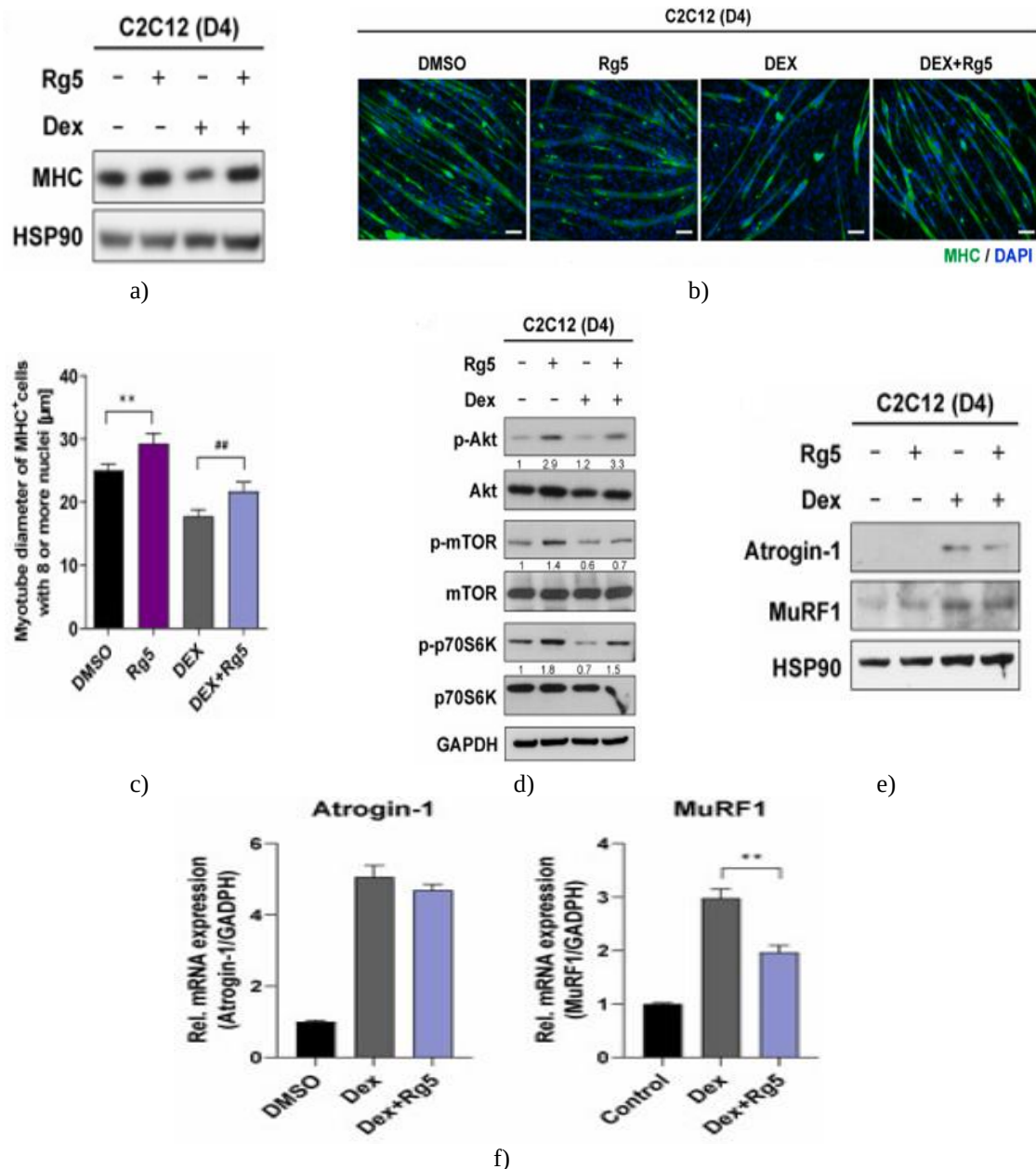


Figure 4. Administration of ginsenoside Rg5 drives muscle hypertrophy via Akt phosphorylation and mitigates atrophy induced by dexamethasone. (a) C2C12 cultures were grown in a differentiation environment for 72 h, after which they were exposed to either the vehicle (DMSO), Rg5 (300nM), or dexamethasone (100 μM) for 24 h. Western blot analysis evaluated MHC expression, utilizing HSP90 as the loading control. (b) C2C12 cultures differentiated for 72 h were subjected to 24-h treatment with vehicle (DMSO), Rg5 (300nM), or dexamethasone (100 μM). Immunostaining was performed to detect MHC, while DAPI was utilized to highlight nuclei (blue). Scale bar = 100μm. (c) Quantitative analysis of the diameter of immunostained C2C12 myotubes from **Figure 4b**. The recorded values indicate the thickness of multinucleated, MHC-positive cells containing over 8 nuclei (N = 4). Results are shown as the mean ± SD. One-way ANOVA was utilized to assess statistical differences. **P < 0.01; Rg5 compared to vehicle (DMSO). ###P < 0.01; DEX + Rg5 compared to DEX. (d) Following 72 h in differentiation media, C2C12 cultures were exposed to vehicle (DMSO), Rg5 (300nM), or dexamethasone (100 μM) for 24 h. Western blotting was conducted to measure the total and phosphorylated expression levels of p70S6K, mTOR, and Akt. Phosphorylated protein quantities were standardized against their corresponding total protein levels and are displayed as fold changes relative to the vehicle (DMSO) control. (e) C2C12 cells cultivated in differentiation media for 72 h were treated with vehicle (DMSO), Rg5 (300nM), or dexamethasone (100 μM) for an additional 24 h. MuRF1 and Atrogin-1 expression levels were assessed via western blotting, with

HSP90 acting as the loading control. (f) C2C12 cultures sustained in differentiation conditions for 72 h were given vehicle (DMSO), Rg5 (300nM), or dexamethasone (100 μ M) over a 24-h period. Relative mRNA levels for MuRF1 and Atrogin-1 were determined by calculating the ratio of each condition's expression relative to the vehicle (DMSO) group. Transcript quantities were standardized to GAPDH levels within corresponding samples. Data reflect the mean $\pm$ SEM. T-tests were utilized to establish statistical significance. **P < 0.01; DEX + Rg5 compared to DEX (DMSO) control.

Subsequently, the influence of Rg5 on the Akt/mTOR cascade—a principal signaling axis governing protein synthesis—was examined. Rg5 administration elevated the overall phosphorylation status of Akt, mTOR, and p70S6K, whereas dexamethasone exposure suppressed these levels. Furthermore, simultaneous application of dexamethasone and Rg5 rescued these phosphorylation states (**Figure 4d**). Notably, when compared to myotubes treated exclusively with dexamethasone, the co-treated cultures exhibited diminished mRNA and protein expression of MuRF1, Atrogin-1, and E3 ligases responsible for degrading muscle-specific proteins (**Figures 4e and 4f**). These findings substantiate that Rg5 drives hypertrophy through the Akt/mTOR signaling network and averts atrophy initiated by dexamethasone.

While extensive investigations into skeletal muscle biology continue, the Food and Drug Administration (FDA) has yet to approve any pharmacological agents designed to enhance muscle strength and function in the context of aging or pathological states. Guided by research from Lee *et al.*, which revealed that black ginseng extract stimulates myogenesis [42], a screening process was conducted to isolate specific compounds capable of combatting muscle atrophy, leading to the identification of Rg5 and its myogenic properties. Literature detailing the influence of ginsenoside Rg5 on muscular metabolism remains scarce [41, 43]. Furthermore, prior to this investigation, no reports had documented the muscle growth or myogenic capacities of Rg5. Consequently, the current research explored the viability of Rg5 as a clinical intervention for muscle wasting. The collected data indicate that Rg5 facilitates muscle regeneration and development by driving myogenesis via p38MAPK phosphorylation, upregulating protein synthesis via Akt/mTOR signaling, and downregulating the expression of atrophy-associated markers, namely MuRF1 and Atrogin-1.

Rk1 and Rg5 are constitutional isomeric ginsenosides. In Rg5, the 21C forms a double bond with 20C, whereas in Rk1, this double bond occurs between 22C and 20C; beyond this, the two molecules share fundamentally similar structures (**Figure 1a**). Despite this structural parity, they elicited distinct myogenic responses (**Figures 1b, 1e**). It is postulated that this variance in double bond positioning is responsible for the divergent myogenic efficacies of these two ginsenosides.

Additionally, it was established that Rg5 drives myogenesis by phosphorylating p38MAPK. To assess if Rg5 might stimulate myogenesis through alternative mechanisms outside the p38MAPK cascade, cultures were co-treated with Rg5 and the p38MAPK inhibitor SB203580. Irrespective of Rg5 administration, pharmacological blockade of p38MAPK suppressed the myogenic response, underscoring the central necessity of the p38MAPK pathway for Rg5-mediated myogenesis (**Figure 3b**). Moreover, the findings demonstrate that Rg5 drives muscle hypertrophy via Akt/mTOR cascade activation while simultaneously curbing glucocorticoid-triggered atrophy. To simulate this wasting condition, C2C12 myotubes were exposed to dexamethasone, a recognized repressor of Akt phosphorylation [15, 44]. The resulting data established that Rg5 counteracts dexamethasone to activate Akt/mTOR signaling, thereby fostering muscle hypertrophy and repressing glucocorticoid-driven muscle degradation.

Within muscle tissues, the transcription factor FoxO3 undergoes activation in response to autophagic stimuli. This FoxO3 activation upregulates the transcription of atrogenes, including MuRF1 and Atrogin-1, a sequence known to be repressed by Akt signaling [26]. In the context of the dexamethasone-stimulated atrophy paradigm, the suppression of Akt phosphorylation by dexamethasone leads to elevated FoxO3 activity and subsequent atrogene expression. Consequently, atrogene levels were quantified to verify that Rg5 administration mitigates dexamethasone-driven atrophy. The findings confirmed an upregulation of atrogene expression following dexamethasone exposure, which was subsequently attenuated by simultaneous Rg5 treatment. These outcomes suggest that Rg5's protective capacity against muscle wasting is mediated not solely by enhanced synthesis of myogenic proteins, but also by the deactivation of FoxO3, which subsequently limits atrogene expression. Nevertheless, relying solely on dexamethasone-treated C2C12 *in vitro* models does not fully encapsulate the complexity of disease-related muscle therapies. Thus, further validation utilizing diverse experimental models is warranted to substantiate these findings.

Conclusion

In conclusion, the present research elucidates the mechanisms by which Rg5 fosters hypertrophy, stimulates myogenesis, and protects against dexamethasone-mediated muscle wasting. The obtained data indicate a clear link between the myogenic properties of Rg5 and p38MAPK phosphorylation. Given prior literature suggesting that p38MAPK phosphorylation augments the binding of MyoD to E2A, immunoprecipitation assays were employed to verify that Rg5's myogenic influence is indeed coupled with this phosphorylation event and the heightened interaction of E2A and MyoD. Additionally, the experimental results established that Rg5 promotes C2C12 cell hypertrophy, as evidenced by expanded myotube diameters, while concurrently mitigating atrophic conditions. These observed effects were directly tied to the phosphorylation and activation of the Akt/mTOR pathway. To the best of current knowledge, this investigation provides the first evidence that Rg5 encourages muscle tissue regeneration, highlighting its potential utility as a pharmacological intervention for treating muscular atrophy and weakness, such as that experienced in cancer cachexia.

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