

Ginkgolic acid regulates myogenic development by influencing the proliferation and differentiation of C2C12 myoblast cells

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Abstract. Ginkgolic acid (GA), isolated from the leaves and seed coats of *Ginkgo biloba*, exerts several biological effects, including antitumor, antibacterial, anti-HIV and anti-inflammatory effects. However, the effects of GA on C2C12 myoblasts remain unclear. The present study assessed cell viability with the MTT assay and evaluated colony formation through crystal violet staining. Flow cytometry was used to analyze apoptosis with Annexin V/7-AAD staining, proliferation with Ki67 staining and cell cycle arrest. Western blotting detected myogenic markers and other relevant proteins. Myotube formation was examined by immunofluorescence, and autophagy was measured using an LC3 antibody-based kit via flow cytometry. The present study showed that treatment of C2C12 cells with GA significantly inhibited their viability and colony formation capacity but did not trigger apoptosis, as indicated by Annexin V/7-AAD staining. However, Ki67 staining indicates that GA exerted dose-dependent antiproliferative effects. Further analysis revealed that GA partially inhibited the growth of C2C12 cells via cell cycle arrest in S phase, highlighting its role in the disruption of cell proliferation. Furthermore, treatment with GA impaired myoblast differentiation, as evidenced by a reduction in the expression of the myogenesis markers, the myosin-heavy chain, myoblast determination protein 1 and myogenin, and suppressed myotube formation. Notably, during C2C12 cell

differentiation, GA promoted apoptosis without affecting cell cycle progression or Ki67 expression. Mechanistically, GA could suppress nuclear extracellular signal-regulated kinase phosphorylation, suggesting that it modulates cell proliferation pathways. Moreover, GA triggered autophagy in differentiated C2C12 cells, as confirmed by elevated LC3 II levels. These findings highlight the multifaceted effects of GA on C2C12 cells.

Introduction

Skeletal muscles are important for maintaining the stability of the human body. Myoblast proliferation and differentiation are complex biological processes during which myoblasts begin to express myogenic factors such as myogenic factor 4, myoblast determination protein and myogenin. During the late stage of muscle differentiation, cells upregulate myosin-heavy chain (MHC) and form a multinuclear structure (1). As they proliferate and differentiate into myotubes during *in vitro* culture, the mouse myoblast cell line C2C12 is widely employed to investigate myoblast differentiation and associated biochemical pathways (2). Using C2C12 cells, a recent study found that after differentiation, genes involved in muscle contraction, autophagy and sarcomeres were more active and that autophagy increased during muscle cell differentiation, as revealed by LC3-I to LC3-II conversion (3). Moreover, chloroquine-mediated inhibition of autophagy can suppress the expression of muscle-specific genes and muscle fiber formation (3), indicating that autophagy has a positive role in muscle cell differentiation and fusion.

Muscle disorders and severe chronic diseases, such as cancer cachexia, HIV/AIDS, chronic heart failure, chronic obstructive pulmonary disease and sepsis, can cause muscle atrophy by decreasing muscle mass and function (4-6). In the short term, muscle atrophy-induced muscle damage causes discomfort because of the inability to move freely, whereas in the long term, it reduces the quality of life and increases mortality. Most cases of muscle atrophy result from decreased protein synthesis or increased protein degradation via the ubiquitin-proteasome system and autophagy (7). The most important markers of muscle atrophy are the ubiquitin ligases MuRF-1 and atrogin-1 (8). Conversely, excessive muscle

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Abbreviations: GA, ginkgolic acid; GM, growth medium; DM, differentiation medium; MHC, myosin heavy chain; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; LC3, microtubule-associated protein 1 light chain 3; SD, standard deviation

Key words: ginkgolic acid, skeletal muscle, proliferation, myogenesis, autophagy, C2C12

formation, characterized by the over-proliferation and differentiation of muscle cells, can lead to muscle-related diseases. For instance, myostatin-related muscle hypertrophy can be attributed to mutations in myostatin, which cause abnormal muscle growth and increased muscle mass and strength (9). Rhabdomyosarcoma is a malignant tumor of the skeletal muscle that disrupts normal muscle formation and leads to abnormal muscle cell proliferation (10). Therefore, research on inhibiting muscle cell proliferation and differentiation is crucial for understanding, preventing and treating these conditions.

Ginkgolic acid (GA), isolated from the leaves and seed coats of *Ginkgo biloba* (11), possesses numerous pharmacological properties, including antitumor, antibacterial, anti-HIV and anti-inflammatory effects (12-15). For example, GA can inhibit lipogenic signaling, thereby delaying pancreatic cancer development (16). GA also activates denosine monophosphate (AMP)-activated protein kinase (AMPK), inhibits colon cancer cell invasiveness (17) and suppresses the migration and metastasis of gastric, breast and lung cancer cells (18-20). By directly binding to the small ubiquitin-related modifier (SUMO)-activating enzyme E1, GA was found to block the formation of the E1-SUMO intermediate, thereby inhibiting SUMOylation (21).

However, the effects of GA on myoblast differentiation remain to be elucidated. The present study examined the effects of GA on the mouse myoblast cell line C2C12 and investigated the underlying molecular mechanisms. Accordingly, it aimed to understand how GA influences muscle cell physiology and provide foundational data for the development of future treatments for muscle-related diseases.

Materials and methods

Cell line and reagents. C2C12 cells were purchased from American Type Culture Collection and cultured in growth medium (GM; DMEM supplemented with 15% fetal bovine serum and 1% streptomycin/penicillin all from Welgene, Inc.) at 37°C, in a humidified incubator (with 5% CO₂) or differentiation medium [DM; GM was replaced with DMEM supplemented with 2% horse serum (Gibco; Thermo Fisher Scientific, Inc.) for 1-3 days]. GA was purchased from MilliporeSigma. Dimethyl sulfoxide (DMSO) was used as the control.

MTT assay. Cells (in 100 µl of GM) were seeded in 96-well plates, cultured for 24 h and then treated with various GA concentrations for 24 and 48 h. Next, 20 µl of MTT stock solution (MilliporeSigma) was added to each well, followed by incubation for 2.5 h at 37°C. The medium was then removed and DMSO was added to each well, followed by cell viability assessment by measuring absorbance at 570 nm on an INNO microplate reader (LTEK Co., Ltd.).

Colony formation assay. C2C12 cells were seeded into six-well plates at a density of 1,000 cells/well and cultured at 37°C for 24 h. The medium was then replaced with fresh medium containing various GA concentrations, followed by culture at 37°C for 4 days until visible colonies containing at least 50 cells were observed. Colonies were visualized using crystal violet staining (cat. no. ab232855; Abcam) according

to the manufacturer's protocol. Briefly, the cells were fixed with 100% methanol at -20°C for 20 min, stained with 2% crystal violet solution for 20 min at room temperature, washed and air-dried. Crystal violet-stained cells were dissolved and absorbance was read at 570 nm on an INNO microplate reader (LTEK Co., Ltd.).

Apoptosis analysis. Apoptosis was evaluated using the Muse Annexin V and Dead Cell Kit (cat. no. MCH100105; Luminex Corporation). This assay kit detects PS on apoptotic cell surfaces using fluorescently labeled (PE) Annexin V, along with the dead cell marker 7-AAD, which stains cells with compromised membranes. C2C12 cells were cultured in six-well plates at a density of 5x10⁴ cells/well and treated with various GA concentrations for 24 or 48 h. The cells were detached using trypsin and resuspended in a fresh medium. Next, 100 µl of Muse Annexin V and Dead Cell reagent was added to the cell suspension, followed by incubation for 20 min at room temperature in the dark. Apoptosis was evaluated using a Guava Muse Cell Analyzer (Luminex Corporation) according to the manufacturer's instructions.

Flow cytometric Ki67 analysis. The Muse Ki67 Proliferation Kit (cat. no. MCH100114; Luminex Corporation) was used to determine the proportion of proliferating cells based on Ki67 expression. Briefly, the cells were harvested and fixed with 1X fixation solution for 15 min at room temperature. Subsequently, the cells were washed with 1X assay buffer, resuspended and treated with the permeabilization solution for 15 min. The cells were then washed and incubated with Muse Hu IgG1-PE (1:20; cat. no. 4700-1669; Luminex Corporation) or Muse Hu Ki67-PE (1:20; cat. no. 4700-1667; Luminex Corporation) antibodies for 30 min, followed by flow cytometry using a Guava Muse Cell Analyzer (Luminex Corporation).

Flow cytometric cell cycle analysis. The Muse Cell Cycle Kit (cat. no. MCH100106; Luminex Corporation) was used for cell cycle analysis. Briefly, cells were centrifuged at 300 x g for 5 min at room temperature and fixed using 70% ethanol at -20°C for 3 h. Next, the cells were stained with the Muse Cell Cycle Reagent (Luminex Corporation) and incubated in the dark for 30 min at room temperature, followed by flow cytometry on a Guava Muse Cell Analyzer (Luminex Corporation).

Western blotting. Western blotting was performed as described previously (22) using the following primary antibodies: anti-MHC (1:200; cat. no. MF20; Developmental Studies Hybridoma Bank), anti-myogenin (1:200; cat. no. F5D; Developmental Studies Hybridoma Bank), anti-myoblast determination protein 1 (MyoD; 1:500; cat. no. 554130; BD Biosciences), anti-poly (ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9542S; Cell Signaling Technology, Inc.), anti-Caspase-3 (1:1,000; cat. no. 9665S; Cell Signaling Technology, Inc.), anti-cleaved Caspase-3 (1:1,000; cat. no. 9664S; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-MEK1/2 (1:1,000; cat. no. 9121S; Cell Signaling Technology, Inc.), anti-phospho-p44/42 MAPK (Erk1/2; 1:1,000; cat. no. 9106s; Cell Signaling Technology, Inc.), anti-phospho-p44/42 MAPK (1:1,000; cat. no. 4695S; Cell Signaling Technology, Inc.), anti-Lamin

B1 (1:1,000; cat. no. 13435s; Cell Signaling Technology, Inc.), anti-LC3B (1:1,000; cat. no. 2775s; Cell Signaling Technology, Inc.), anti- β -actin (1:1,000; cat. no. 4967S, Cell Signaling Technology), anti-MEK-1 (1:500; sc-219), anti-HSP90 (1:500; cat. no. sc-13119) and anti-GAPDH (1:500; cat. no. sc-166574; Santacruz Biotechnology). After washing, the membranes were incubated with the diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; cat. nos. 7074S or 7076S; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein signals were developed and quantified using an Azure imaging system (c280; cat. no. AC2801; Azure Biosystems, Inc.). After initial detection, the membranes were stripped of antibodies using a stripping buffer (cat. no. S2039; Biosesang), followed by reblotting to detect other target proteins. The strips were washed three times with TBS containing 0.05% Tween before immunoblotting.

Immunofluorescence. MHC immunostaining was performed as described previously (22). Briefly, C2C12 cells were seeded at a density of 1×10^4 cells per well using circular glass coverslips (18 mm) placed in 12-well plates. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. Subsequently, cells were blocked with 1% bovine serum albumin (MilliporeSigma) in PBS for 30 min, followed by three washes with 0.2% Triton X-100 in PBS for 5 min each. The cells were then incubated with an anti-MHC (1:100; cat. no. MF20; Developmental Studies Hybridoma Bank) antibody at 4°C overnight. Afterward, the cells were washed three times with 0.2% Triton X-100 in PBS for 5 min each and then probed with a secondary antibody (Fab2-Alexa Fluor 488; 1:500; cat. no. 4408S; Cell Signaling Technology, Inc.) for 1 h at room temperature. The nuclei were counterstained using ProLong Gold Antifade Mountant with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at room temperature, followed by cell examination under a fluorescence microscope (EVOS FL Cell Imaging System; Thermo Fisher Scientific, Inc.). Images were captured using a 20X objective lens.

Fusion index. Nuclei were counted using the ImageJ 1.53a software (National Institutes of Health) and the fusion index was calculated as the ratio between the number of nuclei within each myotube and the total number of nuclei.

Separation of nuclear and cytoplasmic proteins. To examine extracellular signal-regulated kinase (ERK) signaling, NE-PER Nuclear and Cytoplasmic Extraction Reagent (cat. no. 78833; Thermo Fisher Scientific, Inc.) was used to isolate cytoplasmic and nuclear fractions according to the manufacturer's guidelines. Briefly, cells were washed with ice-cold PBS, followed by the addition of cytoplasmic extraction reagent (CER) I to the cell pellet, vortexing and incubation on ice. CER II was then added and the cytoplasmic fraction was isolated by centrifugation at $10,000 \times g$ for 10 min at 4°C. Nuclei-containing pellets were washed with ice-cold PBS, then mixed with nuclear extraction reagent (Thermo Fisher Scientific, Inc.) by vortexing and incubated and centrifuged at $10,000 \times g$ for 10 min at 4°C to obtain the nuclear fraction.

Flow cytometric autophagy assessment. The Muse Cell Analyzer and Muse Autophagy LC3-Antibody Based Kit (MCH200109; Luminex Corporation) were used to assess GA-mediated autophagy, according to the manufacturer's instructions. Briefly, cultured untreated or treated cells were detached and incubated with an anti-LC3-Alexa Fluor 555 antibody and 1X Autophagy Reagent for 30 min in the dark at room temperature. The cells were then resuspended in 1X assay buffer, followed by flow cytometry using a Muse Cell Analyzer (Luminex Corporation). Autophagy induction is presented as the signal ratio between the test and control sample fluorescence.

Statistical analyses. GraphPad Prism version 8.0 (Dotmatics) was used for the statistical analysis. Data are presented as mean $\pm$ standard deviation. The statistical significance of the differences between the GA-treated and control (untreated) groups was determined using Student's unpaired t-test. Statistical differences between the means of multiple groups were compared using one-way analysis of variance, followed by Tukey's multiple comparison test or Dunnett's multiple comparison test if variances were equal. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GA inhibits C2C12 cell viability and colony formation. The present study first performed the MTT assay to evaluate the cytotoxicity of GA on C2C12 cells treated with various GA concentrations (0–100 μ M) for 24 or 48 h (Fig. 1A). Based on the MTT assay results, cell viability decreased significantly to 70 and 77% in cells exposed to 100 μ M GA for 24 and 48 h, respectively, as shown in Fig. 1B (left and right graphs). A colony formation assay was performed to examine the effect of GA on C2C12 cell survival. Crystal violet staining revealed that colonies treated with GA at 20, 50 and 100 μ M for four days were smaller when compared with those in the control group (Fig. 1C). Treatment with GA at 100 μ M reduced absorbance at 595 nm to 82% (Fig. 1D). These findings indicated that treatment with GA significantly affected the viability and colony formation capacity of C2C12.

GA does not influence C2C12 apoptosis. To investigate the mechanism of action of GA in C2C12 cells, its effects on apoptosis were examined. C2C12 cells were treated with increasing concentrations of GA for 24 or 48 h, followed by apoptosis analysis using Annexin V/7-AAD staining. When C2C12 cells were treated with 100 μ M GA for 24 h, the apoptosis rate significantly increased compared to the control, reaching ~2.52%, although more than 95% of the cells remained viable (Fig. 1E, upper panel). Furthermore, we performed western blotting to detect apoptosis-related proteins, including PARP, cleaved PARP, Caspase-3 and cleaved Caspase-3, after 24 h treatment with GA (Fig. S1). The results showed no differences in the expression levels of these proteins between GA-treated and control cells, indicating that this small increase in apoptosis is likely not biologically significant and represents background levels. For the 48 h treatment period, there were no significant changes in apoptosis at all tested concentrations of GA (Fig. 1E, right panel), suggesting that GA does not effectively induce apoptosis in C2C12 cells.

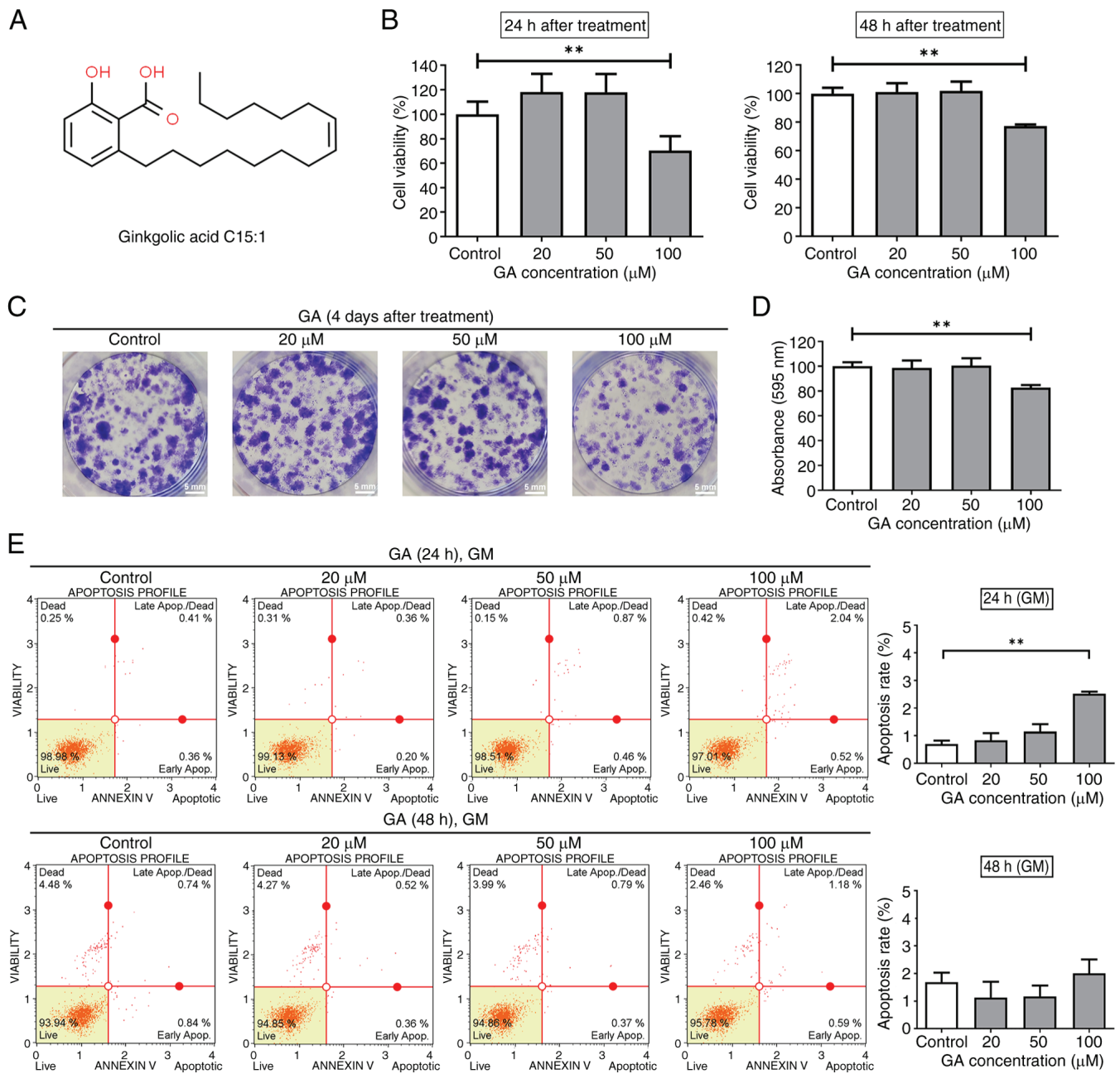


Figure 1. GA decreases C2C12 cell viability and colony formation. (A) The chemical structure of GA. (B) C2C12 cells were treated with GA at indicated concentrations for 24 or 48 h, followed by MTT cell viability analysis. (C) Colony formation analysis. Cells were treated with GA for four days at indicated concentrations. Representative images of crystal violet-stained colonies are shown (scale bar, 5 mm). (D) After four days, crystal violet-stained cells were lysed and the absorbance was measured at 575 nm. (E) C2C12 cells were treated with indicated GA concentrations for 24 or 48 h, followed by Annexin V/7-AAD staining for apoptosis analysis. Data are shown as mean $\pm$ standard deviation. *** $P < 0.01$ vs. the control group (one-way ANOVA with Dunnett's post hoc test). GA, ginkgolic acid; GM, growth medium.

GA suppresses C2C12 cell proliferation. C2C12 cell proliferation was assessed by staining for Ki67, a proliferation marker (Fig. 2A) and quantified using flow cytometry. Following treatment with GA at 100 μM , the proportion of Ki67-positive cells decreased from 89.44-75.72% after 24 h and from 91.5-74.04% after 48 h (Fig. 2A, upper and lower panels, respectively). These results indicated that treatment with GA could exert anti-proliferative effects on C2C12 cells.

GA-mediated cell cycle arrest contributes to C2C12 cell growth inhibition. The cell cycle is crucial for cell growth.

To investigate whether the GA-mediated inhibition of C2C12 cell proliferation was associated with cell cycle arrest, the cells were treated with various GA concentrations for 24 and 48 h and the cell cycle profiles assessed using the Guava Muse Cell Analyzer. Treatment with GA at 100 μM significantly increased the proportion of cells in the S phase after 24 h but not after 48 h (Fig. 2B, upper and lower panels, respectively). Cells in the S phase are mainly engaged in DNA replication and preparation for cell division. These findings indicated that GA-mediated cell cycle arrest partially contributed to the inhibition of C2C12 cell growth.

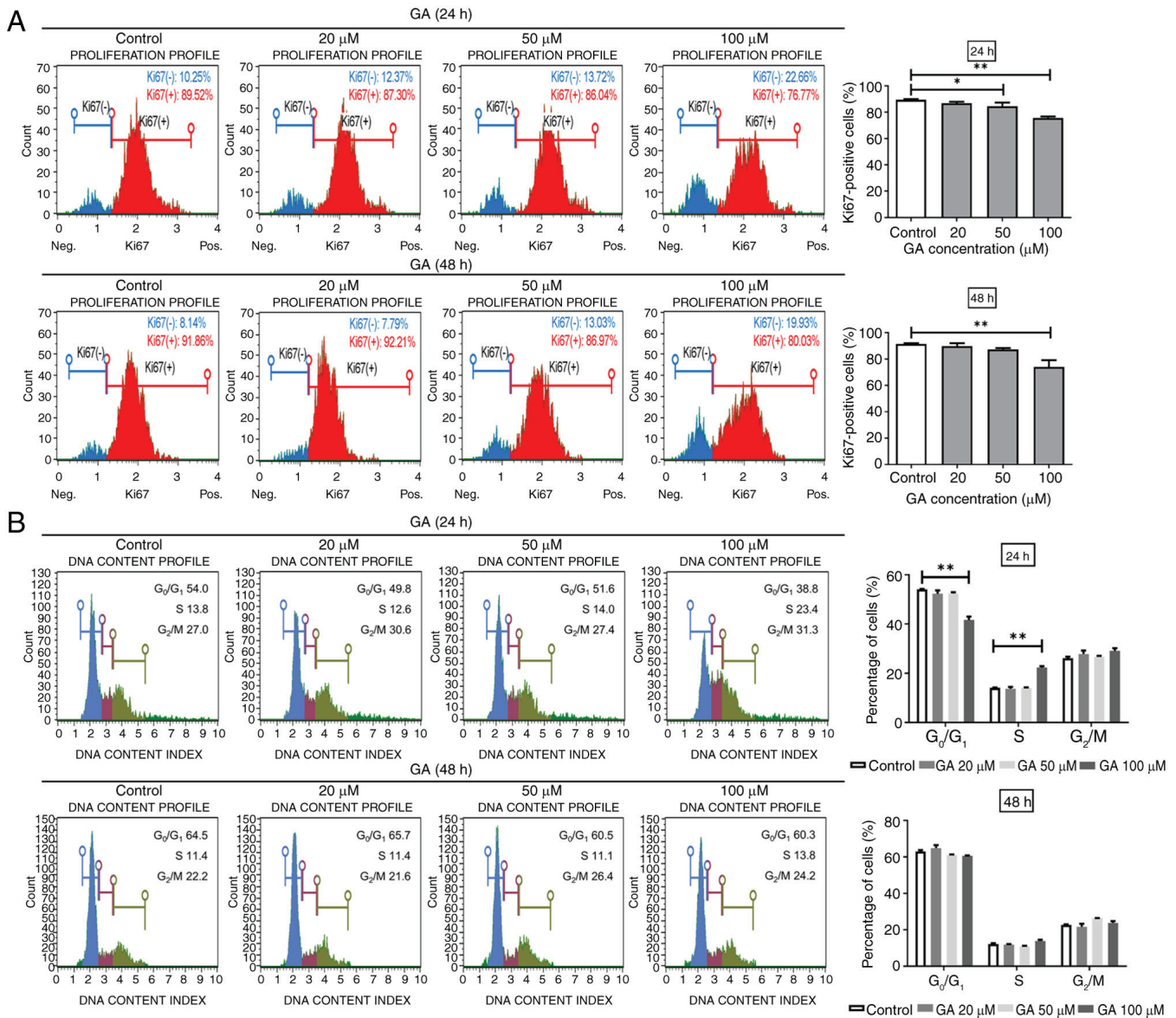


Figure 2. GA inhibits C2C12 cell proliferation and cell cycle. (A) C2C12 cells were treated with GA at 0, 20, 50 and 100 μ M for 24 and 48 h, followed by proliferation analysis using a Muse Ki67 Proliferation kit based on Ki67 expression. Representative results (left panel) and statistical analysis (right panel) are shown. (B) Flow cytometric DNA content analysis of cells treated with GA at 0, 20, 50 and 100 μ M for 24 and 48 h. A representative histogram of the cell cycle distribution (left panel) and a quantification of the cell cycle distribution (right panel) are shown. Data are shown as mean $\pm$ standard deviation of three independent experiments. * P <0.05 and ** P <0.01 vs. the control group (one-way ANOVA with Dunnett's post hoc test). GA, ginkgolic acid.

GA inhibits C2C12 myoblast differentiation. The effects of GA on myogenic differentiation were further investigated by treating C2C12 cells with GA at 20 and 50 μ M for three days, followed by western blotting. Replacing GM with DM for three days significantly enhanced the expression of myogenic markers such as MHC and myogenin (23). Although treatment with GA at 20 μ M did not notably affect the expression of MyoD or myogenin (Fig. 3A), the high GA concentration (50 μ M) markedly reduced MHC, MyoD and myogenin expression compared with the untreated control (Fig. 3B). These results indicated that GA could suppress C2C12 cell differentiation.

GA impairs myoblast fusion. To determine whether GA treatment inhibits myotube formation, we induced the differentiation of GA-treated C2C12 cells for two days, followed by

immunostaining with an anti-MHC antibody and DAPI counterstaining. At 20 or 50 μ M, GA induced cell morphological changes but did not promote cell differentiation (Fig. S2). Compared with control cells, GA-treated C2C12 cells did not form myotubes after two days (Fig. 3C). Quantitative data (fusion index) also revealed that GA reduced the percentage of MHC-positive cells two days after C2C12 differentiation (Fig. 3D). These results indicated that GA significantly inhibited myogenic differentiation in C2C12 cells, as indicated by the reduced levels of MHC-positive myotubes compared to those in control cells.

GA promotes apoptosis but does not affect the cell cycle during C2C12 cell differentiation. Upon establishing that GA exerts toxic effects against differentiated C2C12 cells, flow cytometry was used to investigate the effects of GA on

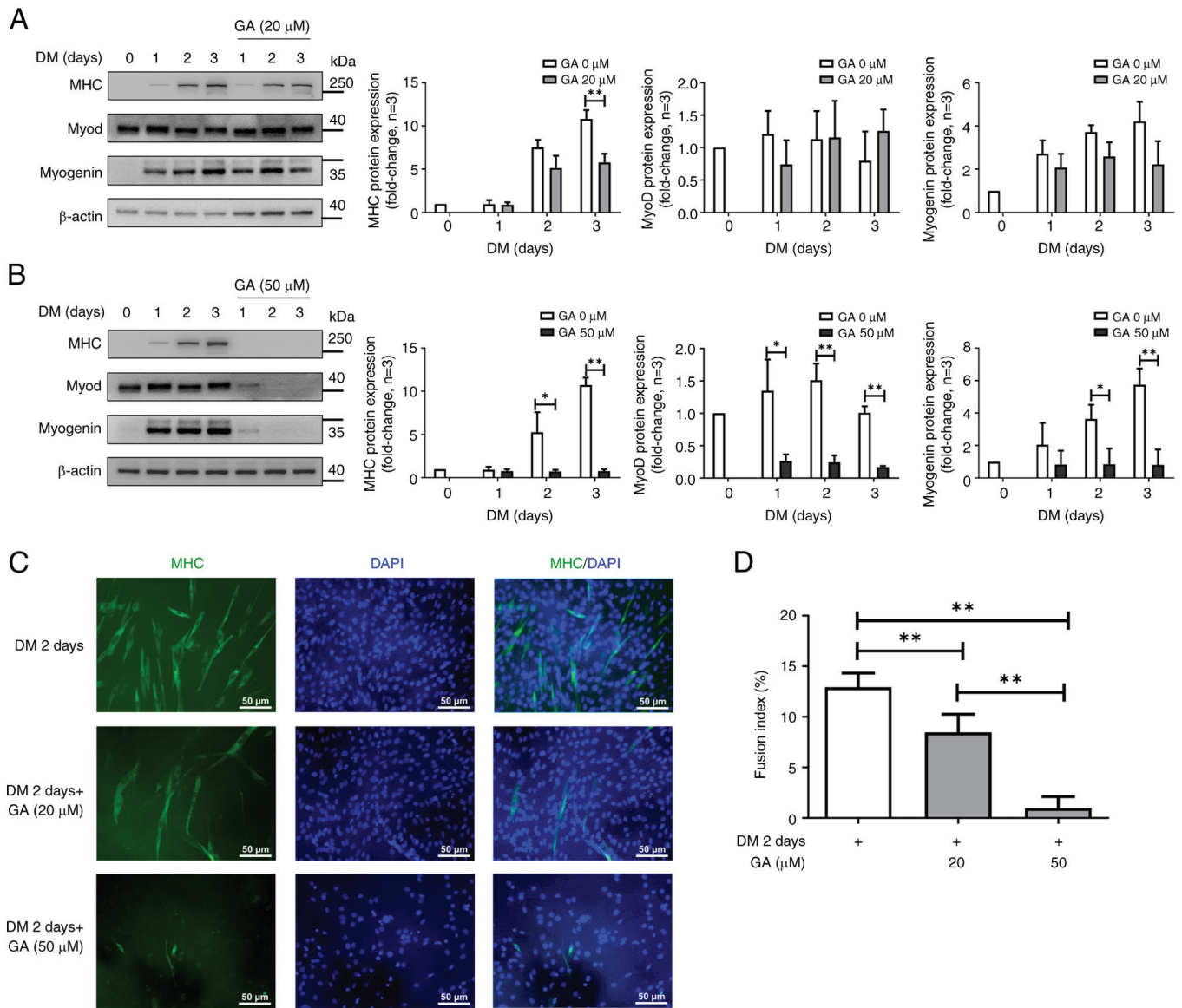


Figure 3. GA regulates C2C12 cell myogenic differentiation. (A) C2C12 cells were treated with GA (20 and 50 μ M) or with DMSO, followed by differentiation induction for three days and western blotting using antibodies against MHC, MyoD, myogenin and β -actin (loading control). (B) Quantification of MHC, MyoD and myogenin protein levels. The bar graphs show relative MHC, MyoD and myogenin protein levels. Relative protein levels were determined based on protein band density using ImageJ. * P <0.05 and ** P <0.01 vs. the untreated group (Student's unpaired t-test). (C) Fluorescence images of C2C12 myotube cells differentiated for two days following treatment with GA (20 and 50 μ M) and staining with an anti-MHC antibody (green, myotube morphology) and DAPI (blue, nuclei). Scale bar, 50 μ m. (D) Fusion index quantification in the control and GA treatment groups. The GA group shows a significant reduction in the fusion index when compared with the control group. * P <0.05 and ** P <0.01 vs. each group (one-way ANOVA with Tukey's post hoc test). Experiments were repeated thrice. GA, ginkgolic acid; DMSO, dimethyl sulfoxide; MHC, myosin heavy chain; MyoD, myoblast determination protein 1; DAPI, 4',6-diamidino-2-phenylindole; DM, differentiation medium.

apoptosis. Cells were cultured in DM with GA at concentrations of 0, 20 and 50 μ M for 24 or 48 h. Treatment of DM with GA for 24 h did not affect the total apoptotic rate of the cells (Fig. 4A, upper panel). However, after 48 h of differentiation, GA significantly increased the total apoptotic rate of cells (Fig. 4A, lower panel). The role of GA in differentiated C2C12 cells was further investigated by flow cytometric cell cycle and Ki67 analyses, revealing no significant differences between GA-treated cells in DM and untreated cells at 24 and 48 h (Figs. 4B and S3). These results indicated that GA could promote apoptosis but does not affect the cell cycle in differentiating C2C12 myogenic cells.

GA affects cell proliferation through Erk phosphorylation. Mitogen-activated protein kinase kinase (MEK) and ERK signaling have been found to promote the proliferation of various cell types, including myoblasts (24). To assess the upstream signaling pathways involved in GA-mediated inhibition of C2C12 cell proliferation, the effects of GA treatment on MEK and ERK phosphorylation, which is crucial for enzyme activation of MEK and ERK, were detected. Compared with untreated C2C12 cells, which exhibited increased levels of ERK phosphorylation after 48 h, treatment with GA strongly decreased ERK phosphorylation in a dose-dependent manner after 48 h without affecting MEK phosphorylation (Fig. 5A). To determine the cellular location of GA-mediated suppression of ERK phosphorylation,

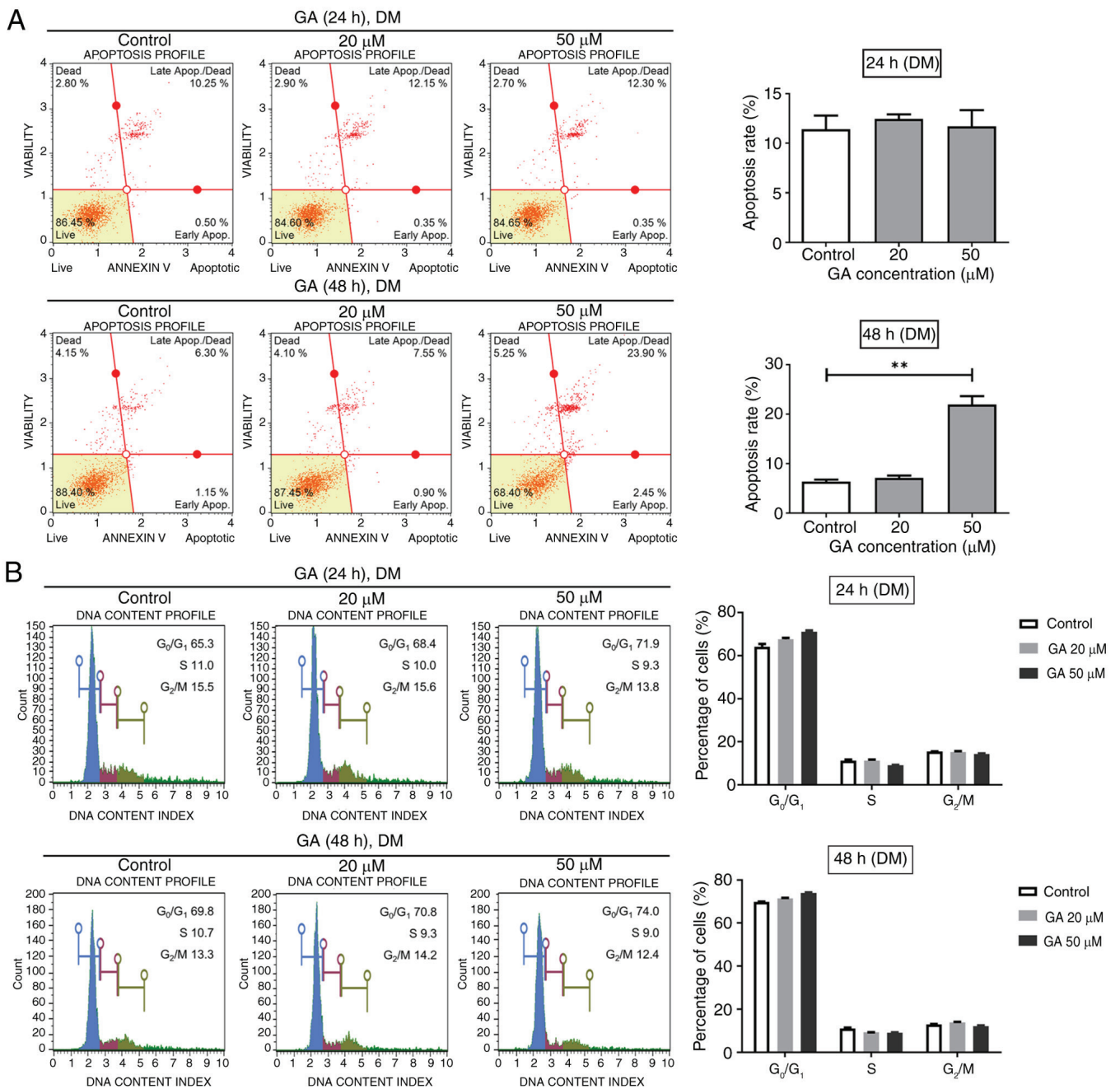


Figure 4. GA induces apoptosis during C2C12 cell differentiation without impacting the cell cycle. (A) Cells were treated with GA at 0, 20, or 50 μ M for 24 and 48 h in DM, followed by flow cytometric apoptosis analysis based on Annexin V staining. Representative results (left panel) and statistical analysis (right panel) are shown. (B) Cells were treated with GA at 0, 20, or 50 μ M for 24 and 48 h, followed by flow cytometric DNA content analysis. A representative cell cycle distribution histogram (left panel) and cell cycle distribution quantification (right panel) are shown. Data are presented as mean $\pm$ standard deviation (SD) of three independent experiments. ** $P < 0.01$ vs. control group (one-way ANOVA with Dunnett's post hoc test). GA, ginkgolic acid; DM, differentiation medium.

nuclear fractions of C2C12 cells treated with GA (0, 20, 50 and 100 μ M) for 48 h were subjected to western blotting. It was found that ERK phosphorylation primarily occurred in the nucleus and that GA suppressed nuclear ERK phosphorylation (Fig. 5B). These results indicated that treatment with GA decreases ERK phosphorylation in a dose-dependent manner without affecting MEK phosphorylation and that in C2C12 cells, it primarily suppressed nuclear ERK phosphorylation.

GA induces autophagy through LC3 activation in differentiated C2C12 cells. Autophagy is a fundamental process that

maintains homeostasis under normal and cellular stress conditions (25). To assess autophagy induction, western blotting and flow cytometry were used to determine LC3 protein levels, a marker of autophagosome formation. LC3 II degradation occurs via lysosome fusion (25). Treatment of differentiated C2C12 cells with GA at 20 and 50 μ M for two days increased LC3 II levels, indicating LC3 I to LC3 II conversion (Fig. 5C). Flow cytometry was used to quantify LC3 levels in differentiated C2C12 cells treated with GA. This analysis revealed an autophagy induction ratio of 1.7 in differentiated C2C12 cells treated with GA at 50 μ M (Fig. 5D and E). However, in C2C12

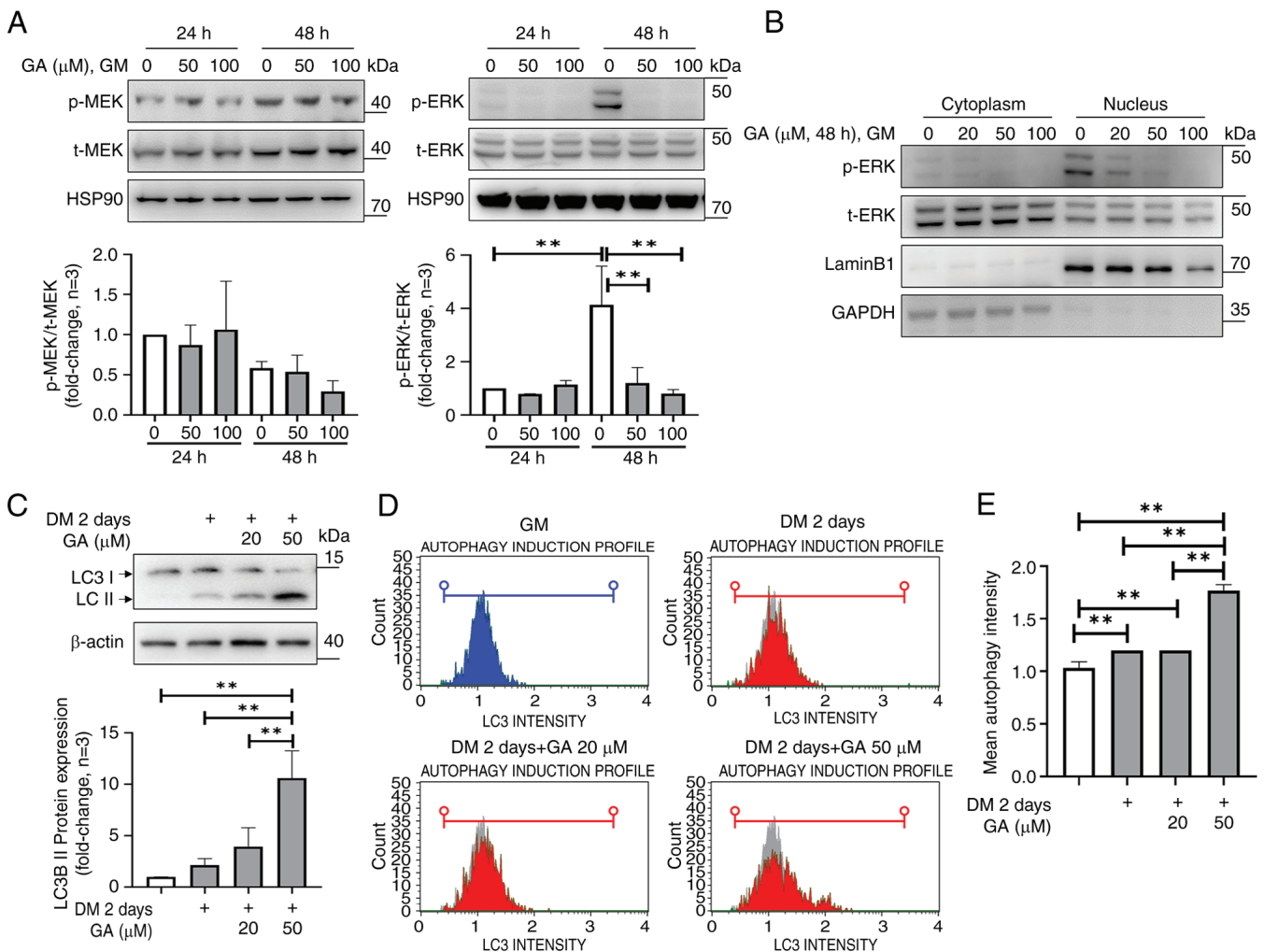


Figure 5. GA inhibits ERK phosphorylation in proliferating C2C12 cells and induces autophagy in differentiated C2C12 cells. (A) Cells were treated with (or without) GA at 50 and 100 μ M for 24 and 48 h in proliferating conditions, followed by immunoblotting for p-MEK, t-MEK, p-ERK and ERK and band intensity quantification using ImageJ (lower panel). HSP90 was used as a loading control. (B) Western blotting of the cytoplasmic and nuclear fractions of C2C12 cells treated with GA at 20 and 50 μ M for 48 h under proliferating conditions. Lamin B1 and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. (C) Western blotting of the levels of the autophagy-related protein, LC3 I/II, in differentiated C2C12 cells treated with (or without) GA for two days. Band intensities were quantified using ImageJ (lower panel). (D and E) Cells were treated with GA at 20 and 50 μ M in differentiation conditions, followed by autophagy quantification using a Muse Autophagy LC3-antibody-based kit on a MUSE cell analyzer. Data are shown as mean $\pm$ standard deviation of three independent experiments. ** P <0.01 vs. each group (one-way ANOVA with Tukey's post hoc test). GA, ginkgolic acid; GM, growth medium; DM, differentiation medium; p-, phosphorylated; t-, total; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase.

cells cultured in GM, treatment with GA at 50 and 100 μ M for 24 or 48 h did not induce LC3 expression differences (Fig. S4A and B). These results indicated that GA induces autophagy and programmed cell death during C2C12 cell differentiation.

Discussion

The present study demonstrated that the treatment with GA reduced the viability and colony formation of C2C12 cells. Although GA suppressed C2C12 cell proliferation, it did not affect apoptosis. GA-mediated cell cycle arrest partly contributed to the inhibition of C2C12 cell growth. Moreover, continued treatment with GA at 50 μ M downregulated MHC, MyoD and myogenin expression under differentiation conditions, thereby substantially delaying myotube formation (fusion index). Additionally, GA suppressed ERK phosphorylation, particularly in the nucleus, highlighting its role in regulating

cell proliferation. Furthermore, in differentiated C2C12 cells, GA triggered autophagy, as evidenced by elevated LC3 II levels, indicating the conversion of LC3 I to LC3 II (Fig. 6).

Myoblast proliferation and differentiation are essential for muscle development, growth and repair. These processes involve myoblast migration, proliferation, differentiation and fusion with mature myofibers. After birth, muscle growth mainly involves an increase in myofiber size, whereas activated satellite cells repair muscle damage (26). The precise regulation of cell proliferation and differentiation is crucial for embryonic and post-embryonic skeletal muscle development. The present study showed that GA inhibited the proliferation and delayed the differentiation of C2C12 cells. In multicellular organisms, cell proliferation is regulated by external growth factors and involves complex processes, including the MAPK pathway. ERK plays a critical role in MEK-ERK signaling, a well-characterized MAPK signaling pathway (27). The present study showed that GA can inhibit C2C12 cell

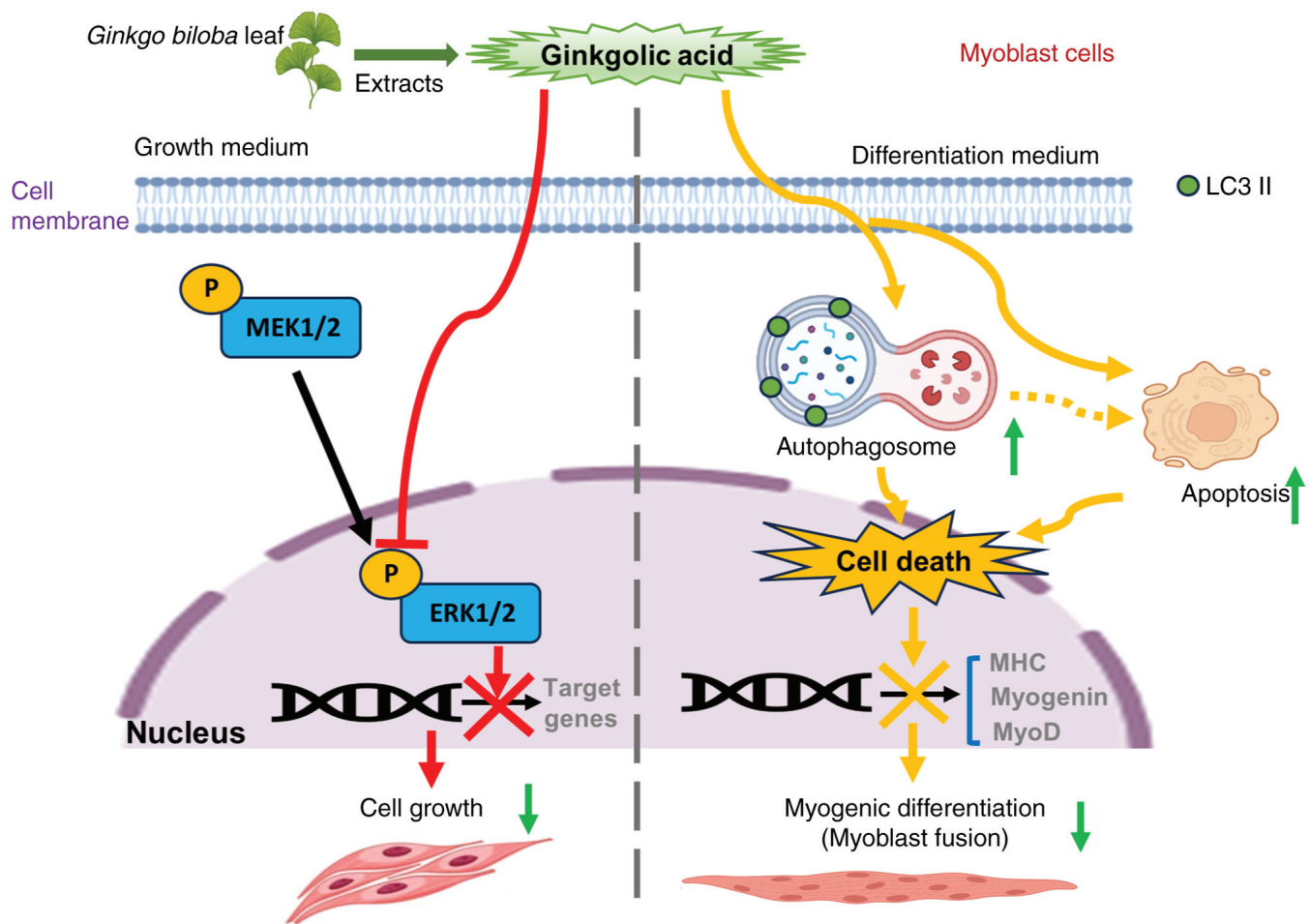


Figure 6. Schematic diagram illustrating the effects of GA on C2C12 cells under two conditions. GA inhibits ERK phosphorylation, particularly in the nucleus, highlighting its role in regulating cell proliferation pathways. Moreover, GA triggers autophagy in differentiated C2C12 cells, as indicated by elevated LC3 II levels, indicating LC3 I to LC3 II conversion. Consequently, treatment with GA can impair myoblast differentiation, resulting in a reduced expression of the myogenesis markers. MHC, MyoD and myogenin and also inhibit myotube formation. GA, ginkgolic acid; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MHC, myosin heavy chain; MyoD, myoblast determination protein 1.

proliferation by suppressing nuclear ERK phosphorylation. Although serum deprivation typically triggers differentiation, this process was enhanced by GA-induced apoptosis and autophagy. Furthermore, as indicated by the reduced MyoD and myogenin expression in differentiated C2C12 cells, GA inhibited myogenesis. MyoD is a key myogenic transcription factor that binds to hundreds of muscle gene promoters and promotes myoblast proliferation (28). By inducing myoblast myogenic differentiation, myogenin induces cell cycle exit and initiates fusion with multinucleated myofibers (29). The present study detected an increased fusion index even after two days of differentiation, which was completely suppressed by GA. Collectively, the data indicated that GA potentially regulates gene expression during C2C12 myogenesis.

The present study explored the role of GA in myogenic development, an area not extensively studied hitherto. Previous research has examined the involvement of GA in various biological processes, but the present study is the first, to the best of the authors' knowledge, to specifically investigate its effects on myogenesis, showing that GA critically affects both myogenesis and general cellular processes. This enhances our understanding of muscle biology and the broader physiological roles of GA. Additionally, the present study is the first

to demonstrate that GA can induce muscle loss in an *in vitro* model, typically involving decreased protein synthesis or increased protein degradation via the ubiquitin-proteasome and autophagy pathways (7). Elevated expression levels of the atrophic markers MuRF-1 and atrogin-1 have been associated with activation of the ubiquitin-proteasome pathway-induced atrophy (8). Substances that inhibit muscle differentiation, such as GA, can be used to treat specific conditions to suppress excessive muscle formation or the growth of certain cancer cells (9,10). Additionally, research exploring GA-mediated inhibition of muscle differentiation can provide valuable insights into the mechanisms and development of treatments for muscle-related diseases, such as sarcopenia and muscle atrophy. For example, the synthetic glucocorticoid analog dexamethasone upregulates muscle-specific E3 ubiquitin ligase genes (8) and has been used to model muscle atrophy (30). Additionally, dexamethasone is widely used to treat various diseases, including cancer and autoimmune disorders (31,32).

Studies indicate that apoptosis regulates the number of muscle cells and mediates myogenesis. In humans and rodents, muscle cell apoptosis leads to skeletal muscle atrophy and sarcopenia (33). Numerous apoptotic factors activate complex and multistep processes of myoblast differentiation (34). Therefore,

elucidating the mechanisms underlying muscle cell apoptosis is crucial to comprehensively clarify skeletal muscle development.

Autophagy is critical for the maintenance of cellular balance in skeletal muscles, particularly during metabolic stress. Insufficient or excessive autophagy can trigger pathological processes leading to muscle weakness and atrophy (35). In C2C12 cells, autophagy is induced during muscle differentiation despite mTOR activation (36). The inhibition of autophagy disrupts myoblast differentiation and promotes apoptosis (37). Increased autophagosome formation or impaired lysosome-autophagosome fusion can cause myopathy (35). Mutations in autophagy genes and dysregulation of the autophagic pathway can substantially contribute to various muscle disorders (35). GA has been shown to activate autophagy, which suppresses cancer cell growth, migration and invasion while triggering cancer cell death (38). As indicated by elevated LC3 II levels, the present study demonstrated that GA could induce autophagy in differentiated C2C12 cells, thereby inhibiting differentiation. Therefore, although optimal autophagy levels are crucial for muscle health and the prevention of debilitating conditions, determining the potential of GA for myoblast treatment as a model of muscle pathology warrants further investigation at functional, histological and molecular levels.

Regarding skeletal muscle cells, GA has been shown to markedly enhance glucose uptake in 3T3-L1 adipocytes and C2C12 muscle cells by activating AMPK signaling (39). Accordingly, GA is a promising therapeutic agent for type 2 diabetes. The present study focused on the effects of GA on the proliferation, differentiation, apoptosis and autophagy of C2C12 cells. Consistent with previous studies, the data showed that *in vitro*, GA regulated not only glucose uptake but also muscle cell proliferation and differentiation.

GA reportedly inhibits SUMOylation by blocking the formation of an E1-SUMO intermediate and directly binding to E1 (21). SUMOylation, a protein modification involving the addition of SUMO molecules, is a key process in various biological and disease contexts (40). SUMOylation involves a cascade of enzymatic reactions catalyzed by E1, E2 and E3 ligases (41). TAK-981 is a novel and selective SUMO E1 inhibitor that affects several cancer cell lines (42,43). Treating multiple myeloma cell lines with TAK-981 and lenalidomide elicited potent synergistic anti-MM activity (44). Moreover, TAK-981 was found to exert anti-leukemic effects mediated via apoptosis induction, cell cycle arrest and immune-independent anti-acute myeloid leukemia activity at nanomolar concentrations (45). Thus, SUMO signaling inhibitors may be beneficial in treating various diseases, including cancer. However, given the potential side effects, such as muscle loss, caution is necessary when using such medications.

Nevertheless, the limitations of the present study need to be addressed. First, it focused solely on C2C12 cells without evaluating other myogenic cell types, such as primary myoblasts or human skeletal myoblast cells. Although C2C12 cells are widely used as models for studying myogenesis, caution is required when extrapolating these results to other cell types or human physiology. Second, although the results suggested that GA treatment affects the proliferation and myogenesis of C2C12 cells, its broad physiological relevance remains uncertain. It is crucial to conduct further studies using diverse myogenic cell types and, more importantly, validate these findings *in vivo*. Additional *in vivo* studies are necessary to fully understand the relevance of the

results of the present study in more complex biological contexts, along with their potential therapeutic implications.

In summary, the present study showed that GA inhibited C2C12 cell proliferation by suppressing ERK signaling and reduced myotube formation by inhibiting myogenesis and activating autophagy. Due to its pharmacological effects, GA has therapeutic potential against various diseases, including cancer. Despite its potential for future drug development and as an alternative treatment in humans, GA causes muscle loss by reducing muscle protein synthesis and enhancing muscle protein breakdown. The present study offered new evidence regarding the molecular mechanisms of GA in C2C12 cells, although this requires further validation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HL designed the experiments and revised the manuscript accordingly. HL and HJ conducted experiments, analyzed the data and wrote the manuscript. HL and HJ confirm the authenticity of all the raw data. Both authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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