ACE activity affects myogenic differentiation via mTOR signaling

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Abstract

Variation in ACE activity affects myogenic differentiation in C2C12 cells. The present study investigated the mechanism by which ACE influences the myogenic differentiation using the ACE-transduced C2C12 cells. Overexpression of ACE induced the down-regulation of myosin heavy chain, a late myogenic marker at 3-5 days after induction of differentiation. ACE-transduced cells exhibited the immature myotubes but an early myoigenc marker (myogenin) was transiently increased at day 1. In ACE-transduced cells, phosphorylation of mTOR and its downstream effector (p70S6K) was suppressed at 2-5 day. However, upstream effector of mTOR (Akt) was transiently suppressed at day 3. Expression of IGF-II mRNA, which is controlled by mTOR, was also down-regulated during the differentiation in ACE-transduced cells. On the other hand, the treatment of cells with captopril, an ACE inhibitor, induced up-regulations of myosin heavy chain and phosphorylated p70S6K. These results suggest that ACE negatively regulates the myotube maturation via impairment of mTOR function.

Key words: ACE; mTOR; IGF-II; differentiation; maturation; myogenesis; C2C12

Introduction

Skeletal muscle exhibits a remarkable capacity to adapt to physiological demands such as growth, training, and injury. The processes by which these adaptations occur are largely attributed to a small population of cells that are resident in skeletal muscle and are referred to as satellite cells, which remain in a quiescence state under normal condition [1]. In response to hypertrophic stimuli, the quiescent satellite cells activate, proliferate, and fuse with the preexisting muscle fibers, or with each other to form the novel muscle fibers [1]. Whereas in atrophied muscle, the number of satellite cells is decreased, and its capacities of proliferation and differentiation are down-regulated [1]. Thus, satellite cells play important roles in skeletal muscle remodeling. Differentiation is one of the events of skeletal muscle remodeling by satellite cells, and has been defined in the in vitro system of myoblast cell cultures [2,3]. This event consists of myogenin expression, cell cycle withdrawal, phenotypic differentiation as marked by contractile expression, and cell fusion to form multinucleated myotube [4], and it involves a large number of intracellular and extracellular mediators in each step [1].

Angiotensin I-converting enzyme (ACE) is a zinc metallopeptidase that generates the vasoconstrictor peptide angiotensin II, inactivates the vasodilator peptide bradykinin, and traditionally thought to play an important role in control of blood pressure through the renin-angotensin system and kallikrein-kinin system [6]. Recently, a large number of studies have suggested that ACE is involved in skeletal muscle

function such as muscle strength and muscle hypertrophy by training [7,8]. Our previous study suggested the role of the satellite cells, which mediate the effect of ACE activity on skeletal muscle function [5].

The treatment of differentiating C2C12 cells, a mouse skeletal muscle satellite cell-derived cell line, with ACE inhibitor induced the up-regulation of myosin heavy chain, and the hypertrophic myotube. On the other hand, overexpression of ACE adversely induced the down-regulation of myosin heavy chain [5]. These results suggested that the variations of ACE activities affected the differentiation of myoblast to myotube. In the present study, we investigated the detailed mechanism by which ACE influences the myogenic differentiation using the ACE-transduced C2C12 cells.

Materials and methods

Materials.

Captopril, low glucose Dulbecco's modified Eagle's medium (DMEM) and mouse monoclonal antibody for myosin heavy chain (MY-32) were purchased from Sigma (St. Lois, MO). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Horse serum (HS) was from Cosmo-bio (Tokyo, Japan). Mouse monoclonal antibody for myogenin, goat polyclonal antibody for β-actin, horseradish peroxidase-conjugated goat anti-mouse antibody and horseradish peroxidase-conjugated rabbit anti-goat antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies for phospho-Akt (Ser473), total-Akt, total-p70S6K and phospho-mTOR (Ser2448), rabbit monoclonal antibody for phospho-p70S6K (Thr389) (108D2) and total-mTOR (7C10), and horseradish peroxidase-conjugated goat anti-rabbit antibody were from Cell Signaling Technology (Beverly, MA).

Cell culture.

Murine C2C12 cells were generously provided by Dr. Tohru Takemasa (University of Tsukuba), and ACE-overexpressed C2C12 cells were generated as previously [5]. Cells were maintained in high glucose DMEM containing 10% heat-inactivated FBS and antibiotics at 37 °C with 5% CO_2. For differentiation, cells were plated at density of 4×10^5 cells per 60 mm dish coated with 0.1% gelatin. After 24 h, the cells were changed into differentiation medium (low glucose DMEM containing 2% HS and antibiotics)

and the medium was changed every 1 or 2 days. To visualize myotubular structures, cells were washed three times in phosphate-buffered saline (PBS) before fixing for 10 min in ice-cold methanol at –20 °C. Cells were stained by Giemsa solution for 1 h and again washed with PBS.

Western blot analysis.

Cells were washed twice with ice-cold PBS, and lysed with lysis buffer composed of 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM NaF, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol, and protease inhibitors (0.57 mM phenylmethylsulfonyl fluoride, 3 μg/ml Leupeptin, 3 μg/ml Pepstatin A, and 0.4% Aprotinin). Lysates were incubated on ice for 30 min followed by 20 min of centrifugation at 14000 × g, and protein concentrations of the supernatants obtained were determined by the Bradford method. Each sample (10-15 μg of cell protein) was separated on 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% skim milk in Tris-bufferd saline containing 0.1% Tween 20 (TBST), and then incubated with primary antibodies overnight at 4 °C. The membranes were washed with TBST and further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washed with TBST, immunocomplexes were visualized on X-ray film by chemiluminescence according to manufacture's instructions (GE Healthcare, Piscataway, NJ). Band intensities of immunoblots were assessed by using ImageJ (National Institute of Health, Bethesda, MD).

Reverse transcription-Polymerase chain reaction (RT-PCR).

Cells were washed twice with ice-cold PBS, and total RNAs were extracted using RNA-Bee (Tel-test, Friendswood, TX). DNase I-treated RNAs were reverse transcribed using TaKaRa RNA PCR kit according to manufacture's instruction (TaKaRa-Bio, Otsu, Japan). The cDNA was diluted in a 1 : 4 ratio, and 2 μl was used for PCR. The primers for IGF-II used were: forward 5'-TCAAGCCGTGCCAACCGTCGC-3'; reverse 5'-CTCCGAAGAGGCTCCCCCGTG-3'. The primers for GAPDH (forward 5'-GTGGCAAAGTGGAGATTGTTGCC-3'; reverse 5'-GATGATGACCCGTTTGGCTTC-3') were used as an internal control. Each PCR regime involved a 94 °C, 5 min initial denaturation step followed by either 25 cycle (GAPDH) or 35 cycle (IGF-II) at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, followed by a final amplification step of 5 min at 72 °C. The PCR products were separated by electrophoresis on 2% agarose gel.

Statistical analysis.

Data are expressed as means ± SD. Comparisons between study groups were made using unpaired *t*-tests, with a *P* value of < 0.05 being considered statistically significant.

Results

Overexpression of ACE suppresses the myogenesis by the impairment of myotube maturation.

Previously, we had produced the ACE-overexpressed C2C12 cells in order to investigate the effect of increase in cellular ACE activity on the myogenic differentiation. In ACE-transduced cells, myosin heavy chain (MyHC) was down-regulated when compared with control cells [5]. Similar to the previous results, the ACE-transduced cells contained less MyHC at day 3 and the suppressed level of MyHC was maintained for up to day 5 after the induction of differentiation compared with the control cells (Fig. 1A). Unexpectedly, the expression of myogenin, the early myogenic marker, was up-regulated in the ACE-transduced cells at day 1 (Fig. 1B), suggesting that the overexpression of ACE did not suppress the initiation of myogenic differentiation. In morphological observation, the control cells exhibited a large number of thickened and branched myotubes with clustered nuclei at day 5 (Fig. 1C, a), while the sizes of myotubes were smaller, and the fusions of cells to form thickened myotubes were less identified in the ACE-tranduced cells (Fig. 1C, b). Collectively, these results suggest that the overexpression of ACE does not suppress the beginning of differentiation program, however, the processes of further fusion and protein synthesis that gives rise to mature myotube size are impaired in these cells.

Overexpression of ACE suppresses the mTOR signaling and the expression of IGF-II.

To investigate the mechanism of the down-regulation of MyHC in ACE-overexpressed C2C12 cells, we focused on the signaling molecules of Akt and mammalian target of rapamycin (mTOR) required for the skeletal muscle development, and examined the phosphorylation of these signaling molecules. Although significant suppression in phosphorylation of Akt on Ser473 in the ACE-transduced cells was observed only at day 3 (Fig. 2A), decreased phosphorylation of Ser2448 in mTOR, considered as a downstream effecter for Akt, was already identified at day 2 and remained for up to day 5 (Fig. 2B). Phophorylation of ribosomal protein S6 kinase (p70S6K) on Thr389, required as a downstream effector of mTOR, was suppressed from day 2 to day 5 in ACE-overexpressed cells (Fig. 2C). These results suggest that the down-regulation of MyHC by the overexpression of ACE was due to the impairment of mTOR signaling, but Akt was unlikely to be involved in this process. As insulin-like growth factor II (IGF-II) transcription in skeletal myogenesis is controlled by mTOR independent of its kinase activity [9], the present study investigated whether the impairment of mTOR activation affected the expression of IGF-II mRNA during the myogenic differentiation. In semiquantitative RT-PCR analysis, the decrease in the expression levels of IGF-II mRNA were clearly identified from day 2 to day 5 in the ACE-transduced cells compared with the control cells (Fig. 2D), suggesting that IGF-II mRNA expression was controlled by ACE via the regulation of mTOR activity.

ACE inhibitor induces the acceleration of myogenesis by the activation of mTOR signaling.

Previously, we demonstrated that the decrease in extracellular ACE activity by captopril, an ACE inhibitor, induced the up-regulation of MyHC in the myogenic differentiation [5]. To confirm that ACE activity affect myogenic differentiation through mTOR signaling, p70S6K phosphorylation and IGF-II mRNA expression was examined in C2C12 cells treated with captopril. Similar to the previous results, treatment with 1000 μM captopril induced the up-regulation of MyHC compared with non-treatment (Fig. 3A). Furthermore, the increased levels of p70S6K phophorylation (Fig. 3A) and IGF-II mRNA expression (Fig. 3B) were also observed. These results suggest that mTOR signaling also plays a role when myogenic differentiation is stimulated by ACE inhibitor.

Discussion

The present study demonstrated that overexpression of ACE suppressed myogenic differentiation. MyHC was down-regulated and maturation of myotube was impaired in ACE-overexpressed C2C12 cells. Furthermore, suppression of myogenic differentiation by ACE was accompanied with decreased mTOR signaling. mTOR is a large protein with Ser/Thr kinase activity, which regulates mammalian cell growth and proliferation by mediating signal transduction of multiple signaling [10]. Phosphorylation of Ser2448 on mTOR has been suggested to play a key role in regulating mTOR function [11]. Decreased level of phosphorylation on Ser2448 of mTOR in ACE-transduced cells indicates the impairment of signal transduction through mTOR. Although we could not directly determine the kinase activity of mTOR, decreased level of p70S6K phophorylation could be regarded as the suppressed kinase activity of mTOR in ACE-transduced cells. This takes into consideration that mTOR directly phosphorylates p70S6K on Thr389 [12]. Previous studies have shown that mTOR signaling plays essential roles in myogenic differentiation and skeletal muscle hypertrophy [13-17], and the myogenic differentiation is thought to consist of two stages. It begins as formation of nascent myotubes by myoblast-myoblast fusion, and maturation of myotubes by further fusion and increase in protein synthesis follows. The formation of mature myotubes requires the kinase activity of mTOR and a previous study has shown that C2C12 cells supported by a kinase-inactive mTOR form nascent myotubes, but not mature myotubes [18]. Consistent with the proposed role of mTOR in

myogenic differentiation, myotubes of ACE-transduced cells in the present study exhibited immature phenotyoe i.e. the size of myotubes was smaller and the number of nuclei per myotube was decreased. Therefore, the present findings suggest that gain of ACE activity impairs mTOR signaling to suppress myogenic differentiation in ACE-overexpressed C2C12 cells.

Interestingly, the expression of myogenin was transiently enhanced at day 1 in ACE-overexpressed cells, while the changes in phosphorylation of mTOR and p70S6K were not significant. The down-regulations of these phosphorylated proteins became significant from day 2, when myogenin expression reached the abundant level. These temporal relations imply distinct functions of ACE to stimulate initiation of differentiation, independent of mTOR signaling, and to inhibit myotube maturation through mTOR signaling.

Autocrine actions of IGF-II are suggested to be important for the myogenic differentiation of satellite cells including C2C12 cells in culture [19,20]. Furthermore, recent study has shown that the autocrine IGF-II transcription required for skeletal myocyte differentiation is regulated by mTOR activity, which is independent of its kinase activity [8]. In our study, the decrease in expression of IGF-II mRNA by the overexpression of ACE was clearly identified from day 2, when decreased level of phophorylation of mTOR was also observed, suggesting that the expression of IGF-II mRNA was influenced by the level of mTOR activation. Taken together, these results suggest that the effect of ACE on the myogenic differentiation involves the changes in mTOR signaling and mTOR-induced IGF-II functions.

In the present study, the phoshorylation of Ser2448 in mTOR was suppressed in ACE-transduced cells. Although it has been shown that Akt phosphorylates this site [11,21], we did not identify the parallel decrease in the level of phophorylation of Akt and mTOR in the ACE-transduced cells. Therefore, we speculated that the overexpression of ACE targeted the impairment of mTOR activity, and Akt was unlikely to be involved in the regulation of mTOR activity by ACE. Reduction of Akt phosporylation in ACE-transduced cells became statistically significant at day 3, when expression of IGF-II mRNA in control cells appeared to reach the maximal level and it was suppressed by ACE overexpression. Since Akt is stimulated, i.e. phosphorylated, by IGF and this pathway plays a critical role for IGF's myogenic signaling [9], one possible explanation for the transient suppression of Akt phosphorylation in ACE–transduced cells on day 3 is that Akt activity is affected by the action of mTOR through changes in IGF-II expression.

The mechanism by which ACE suppressed the activity of mTOR remains unclear. However, previous studies have reported that angiotensin II, generated by ACE, induces muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway [22,23], and that bradykinin, degraded by ACE, is involved in glucose uptake in skeletal muscle [24,25]. A large number of studies have shown that the activity of mTOR is regulated by nutrients, growth factors, and cellular metabolism [10]. Furthermore, recent study has indicated that mTOR forms a complex with raptor, the regulatory associated protein of mTOR, in response to the nutrient-dependent signal, and that mTOR/raptor phosphorylates p70S6K on Thr389 [12]. On the other hand, the

signals of growth factors and insulin are thought to mediate the phosphorylation of Akt on Ser473 through a complex of mTOR with rictor, instead of raptor [12]. Possibility that nutrient and growth factor signals require different partner of mTOR suggests the reason why effect of ACE overexpression on Akt phophorylation was different from that of mTOR and p70S6K. The present study suggests that ACE affects the availability of nutrients leading to the change in the activity of mTOR. Further study to elucidate our hypothesis concerning the relationship between the control of nutrients by ACE and mTOR activity is required.

In conclusion, the present study demonstrates that variations in ACE activities affect myogenic differentiation via mTOR signaling pathway. Although ACE may possibly cause the changes in nutrient availability to affect mTOR activity, detailed mechanism remains unclear and to be elucidated. These studies may shed light on the novel role of ACE in the skeletal muscle.

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Figure legends

Fig. 1. Overexpression of ACE suppresses the myogenic differentiation by the impairment of myotube maturation. Control C2C12 cells (C2C12-control or C) and ACE-transduced cells (C2C12-ACE or A) were cultured in differentiation medium (DM). Each DM was changed at days 1 and 3 after induction of differentiation, and cell lysates were harvested. Western blot analysis of cell lysates was performed to determine the expression levels of MyHC (A) and myogenin (B). Representative blots pattern was shown. The results were expressed as relative values to β-actin expression, and means of four different cultures. Black and white bars represent C2C12-ACE and C2C12-control cells, respectively. $*P < 0.05$, $**P < 0.01$ versus C2C12-control. Morphological analyses of both C2C12-control (a) and C2C12-ACE (b) myotubes were performed with Giemsa staining at day 5 after induction of differentiation (C). Scale bars, 200 μm.

Fig. 2. Overexpression of ACE suppresses the mTOR signaling and the expression of IGF-II mRNA. Control C2C12 cells (C2C12-control or C) and ACE-transduced cells (C2C12-ACE or A) were cultured in DM. Each DM was changed at days 1 and 3 after induction of differentiation, and cell lysates and total RNAs were harvested. Western blot analysis of cell lysates was performed to determine the phosphorylation (depicted as “p-“) levels of Akt (Ser473) (A), mTOR (Ser2448) (B) and p70S6K (Thr389) (C). Representative blots pattern was shown. The results were expressed as relative values to

each total protein (depicted as “t-“) expression, and means of four different cultures. Black and white bars represent C2C12-ACE and C2C12-control cells, respectively. *P < 0.05, **P < 0.01 versus C2C12-control. RT-PCR analysis of RNAs was performed to determine the expression level of IGF-II mRNA. The bands of GAPDH were shown as internal controls (D).

Fig. 3. ACE inhibitor induces the acceleration of myogenic differentiation by the activation of mTOR signaling. C2C12 cells were cultured in either DM alone (-) or with 1000 μM captopril (+). Each DM was changed at days 1 and 3 after induction of differentiation, and cell lysates and total RNAs were harvested. Western blot analysis was performed to determine the levels of MyHC expression and p70S6K (Thr389) phosphorylation (p-p70S6K) (A). The blots of total p70S6K (t-p70S6K) and β-actin were shown as internal controls. RT-PCR analysis of RNAs was performed to determine the expression level of IGF-II mRNA. The bands of GAPDH were shown as internal controls (B).

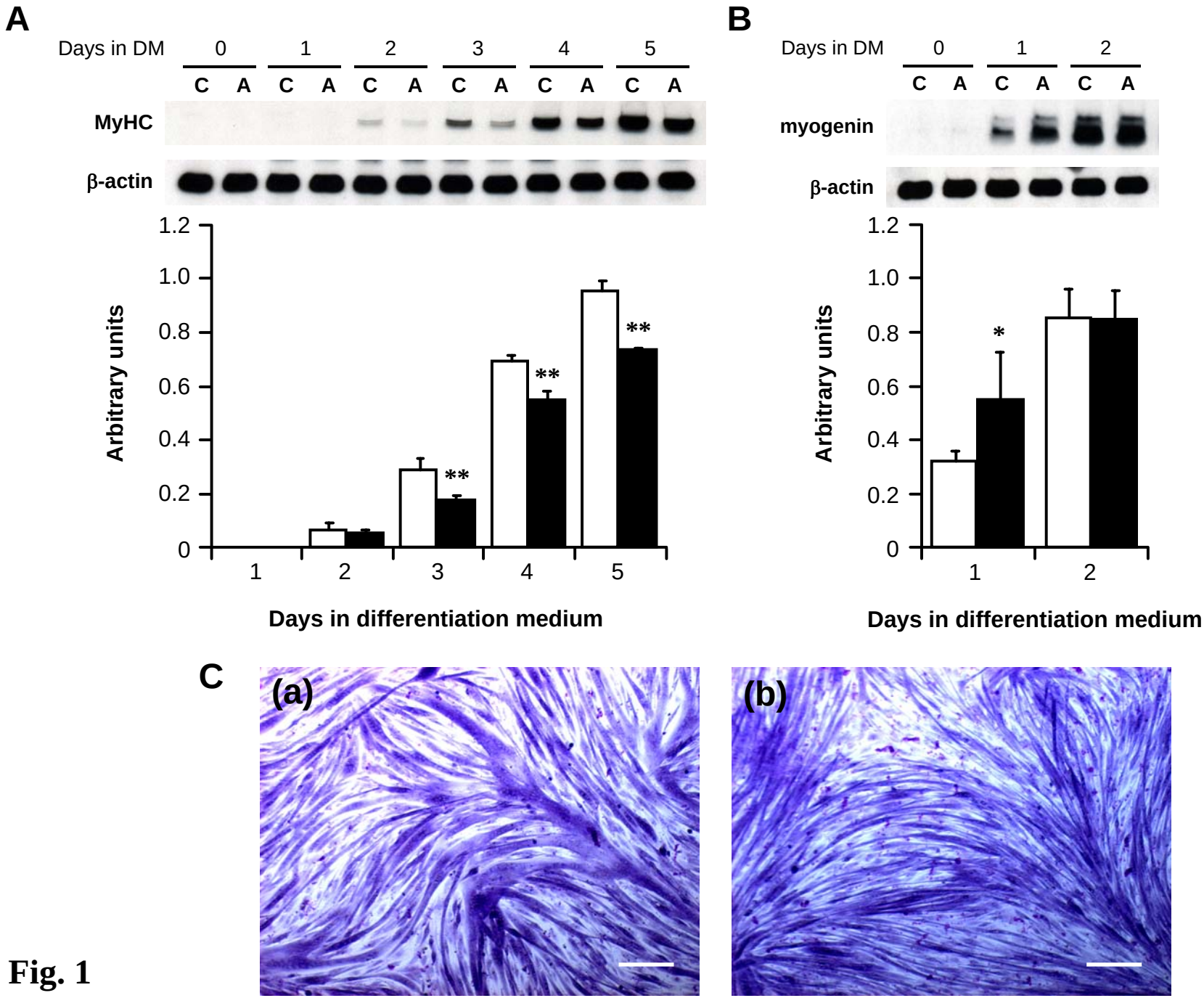
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Fig. 1

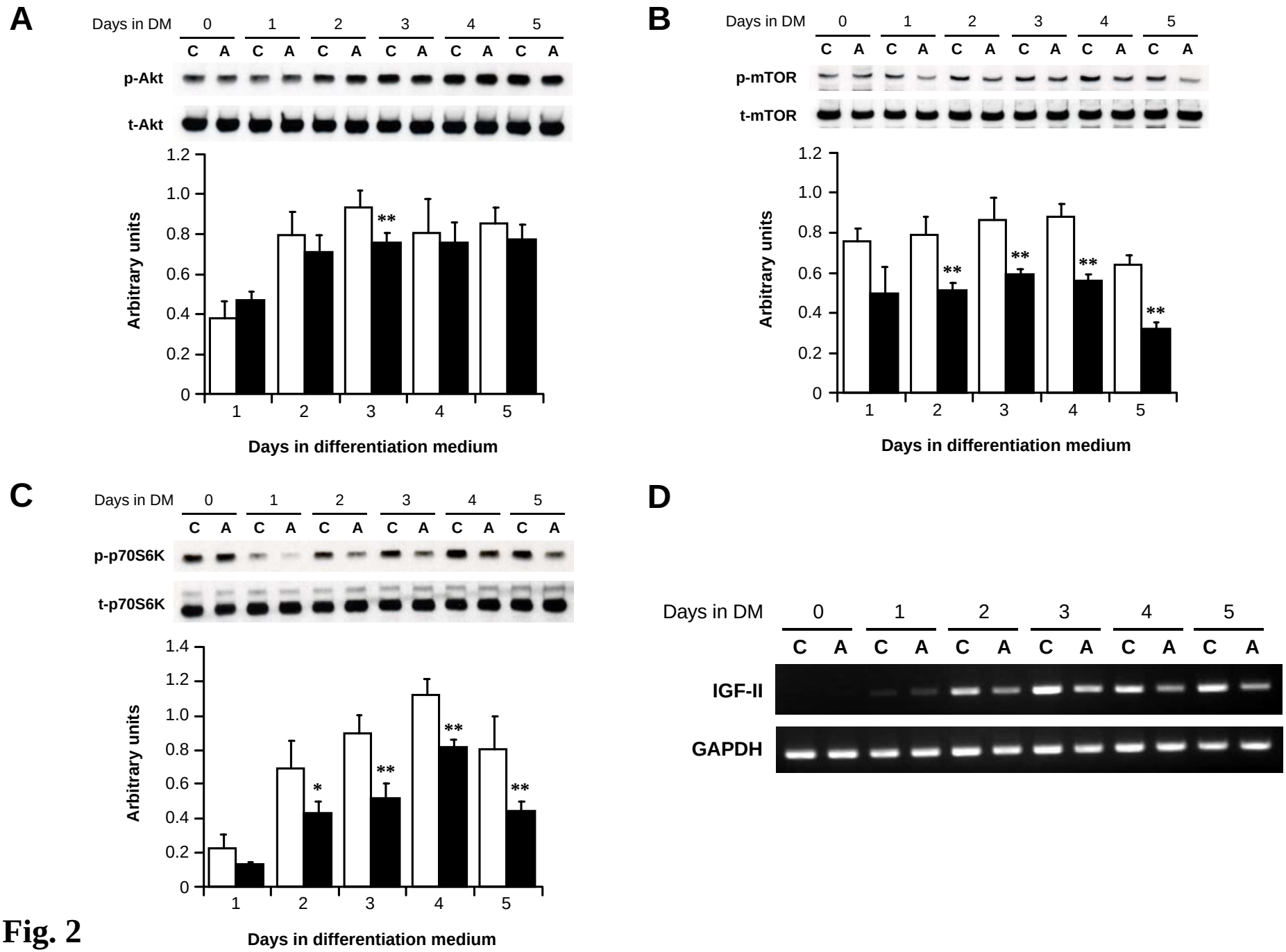
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Fig. 2

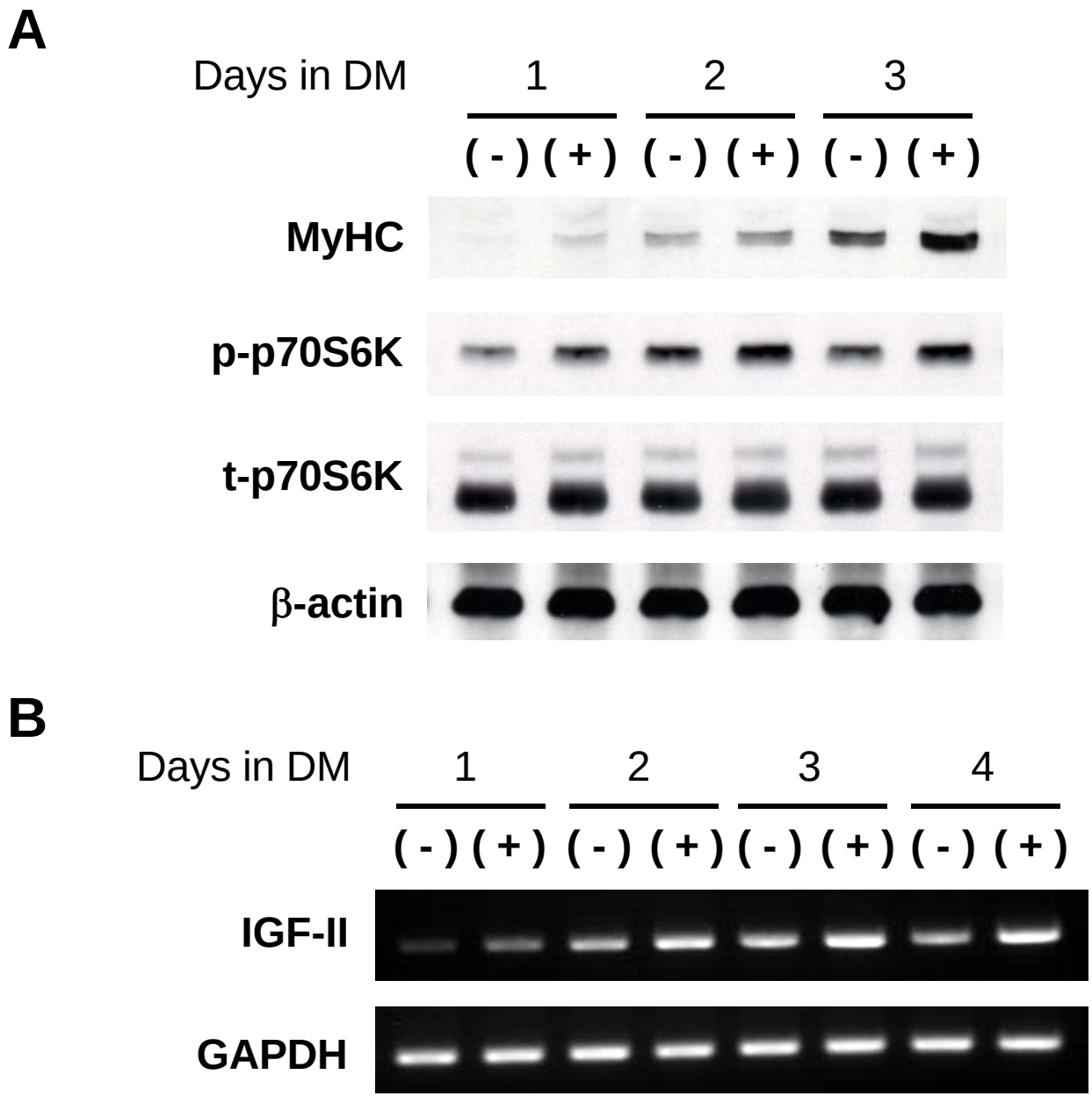
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Fig. 3