Enhancement of vitamin C-induced myogenesis by inhibition of extracellular signal-regulated kinase (ERK) 1/2 pathway

Xiajie Fu, Tohru Matsui, and Masayuki Funaba*

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Running title: Modulation of myogenesis by ERK and vitamin C

*Corresponding author: Masayuki Funaba, Ph.D.
Division of Applied Biosciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan
Tel.: +81-75-753-6055
Fax: +81-75-753-6344
E-mail: funaba.masayuki.8w@kyoto-u.ac.jp

Highlights

Endogenous ERK1/2 activity negatively regulates myogenesis

Inhibition of ERK activity greatly enhances vitamin C-stimulated myogenesis

Inhibition of p38 MAP kinase represses myogenesis in the presence of vitamin C

The role of endogenous JNK activity is similar to that of endogenous ERK1/2 activity

Abstract

Myogenesis is a complex process that is regulated by a variety of factors. We have previously shown that vitamin C and mild endoplasmic reticulum stress synergistically enhance myogenesis. The present study evaluated the effects of vitamin C (ascorbic acid (AsA) and AsA 2-phosphate (AsAp)) and extracellular signal-regulated kinase (ERK) 1/2 pathway on myogenesis. Treatment with U0126, an inhibitor of MEK1/2 that phosphorylates and activates ERK1/2, during the differentiation, increased the mRNA levels of *Myod* and *Myog* with an increase in the protein level of myosin heavy chain (MYH)1/2. Treatment with AsA or AsAp alone had minimal effects on myogenesis in C2C12 cells. However, combination treatment with vitamin C and U0126 greatly enhanced myogenesis; the number of thick and long myotubes was increased, and the expression of MYH1/2 was also increased. PD98059, another MEK1/2 inhibitor, also enhanced myogenesis in combination with vitamin C. These results indicate that relief of endogenous ERK1/2 activity enhances vitamin C-mediated myogenesis, suggesting a functional interaction between endogenous ERK1/2 activity and vitamin C. In addition, inhibition of p38 mitogen-activated protein kinase repressed myogenesis in the presence of vitamin C. Thus, vitamin C is a conditional factor that modulates myogenesis.

Key words: myogenesis, vitamin C, ERK1/2, p38

1. Introduction

The skeletal muscle is mainly composed of myofibers and is responsible for skeletal movement through contractility. Muscle contraction is achieved by myofibrillar proteins, including actin, myosin heavy chain (MYH), myosin light chain (MYL), troponin, and tropomyosin. Myofibers are formed via the following sequential differentiation steps: commitment of mesenchymal stem cells to myogenic lineage cells, myoblast proliferation, fusion of myoblasts into multinucleated myotubes, and myotube maturation [1-3]. Myogenesis is primarily controlled by basic helix-loop-helix transcription factors named as myogenic regulatory factors (MRFs), i.e., myogenic factor 5 (MYF5), myoblast determination protein 1 (MYOD), myogenin (MYOG), and myogenic regulatory factor 4 (MRF4). Various molecules, including internal and external factors, are involved in the regulation of myogenesis by modulating the expression and activity of MRFs [1-3].

Mitogen-activated protein (MAP) kinase signaling regulates diverse (patho-) physiological processes via the modulation of cell proliferation and motility [4]. The conventional MAP kinase consists of three modules, namely the extracellular signal-regulated kinase (ERK) 1/2, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) pathways [4]. Previous studies have shown that the ERK1/2 pathway is involved in the regulation of the proliferation and differentiation of C2C12 myoblasts, which are frequently used as a cell model to evaluate myogenesis; however, the results are inconsistent [5-8]. Jo et al. [5] examined the effect of an MEK1/2 inhibitor (PD184352 or U0126), which represses phosphorylation and activation of ERK1/2, on myogenesis; protein levels of MYH were observed to be lower in C2C12 cells treated with inhibitors than those in control cells [5]. Knockdown of *Erk2* led to a decrease in myotube formation induced by culture with reduced mitogens [6]. These results suggest that endogenous ERK1/2 activity is required for proper myogenesis in C2C12 cells. In contrast, PD98059, another MEK1/2 inhibitor, potentiated an increase in

myotube formation, indicating a negative regulation of myogenesis by endogenous ERK1/2 activity [7]. Furthermore, the ERK1/2 pathway induced positive and negative regulation of myogenesis [5, 8].

Vitamin C, a generic term used for all compounds that show the biological activity of ascorbic acid (AsA), increased myogenesis; treatment with AsA 2-phosphate (AsAp, a stable form of AsA) slightly increased MYOG protein levels and promoted myotube maturation [9, 10]. In addition, we have recently revealed that induction of mild endoplasmic reticulum (ER) stress prior to the differentiation stimulation of myogenesis greatly enhanced vitamin C-induced myogenesis [11]. These results prompted us to hypothesize that myogenesis is rigorously regulated through functional interactions between internal factors, such as cell response and signaling, and external factors, such as diet. The present study explored the role of endogenous ERK1/2 pathway as an internal factor and its relationship with vitamin C-induced myogenesis in C2C12 myogenic cells.

2. Materials and Methods

2.1. Materials

The following reagents were purchased: U0126, PD98059, SB203580, ascorbic acid (AsA), and ascorbic acid 2-phosphate (AsAp) were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan); SP600125 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA); antibodies against β-actin (#4967), ERK (#9102), JNK (#9252), p38 (#9212), phospho-ERK (Thr202/Tyr204) (#9101), phospho-JNK (Thr183/Tyr185) (#9251), and phospho-p38 (Thr180/Tyr182) (28B10) (#9216) were obtained from Cell Signaling Technology (Danvers, MA, USA); antibody against MYH1/2 (A4.1025) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA); Chemi-Lumi One Ultra was purchased from Nacalai Tesque

(Kyoto, Japan).

2.2. Cell culture

C2C12 myoblasts were obtained from RIKEN BioResource Research Center (Tsukuba, Japan). Cells were grown in a growth medium, namely Dulbecco's modified Eagle's medium (DMEM), containing heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C under a humidified 5% CO_2 atmosphere. At day 1 after reaching the confluence (day 0), C2C12 myoblasts were stimulated to induce differentiation from myoblasts to myotubes by culture in differentiation medium consisting of DMEM with 2% horse serum supplemented with antibiotics. Cells were treated in differentiation medium with or without AsA or AsAp (100 μM or 200 μM) and the indicated concentrations of inhibitors of the MAP kinase pathway (U0126, PD98059, SB203580, and SP600125) from day 0 to day 4. Dimethyl sulfoxide was used as the vehicle control for the inhibitors.

2.3. RNA isolation and real-time RT-quantitative PCR (RT-qPCR)

Total RNA isolation and RT-qPCR were performed as previously described [12]. The primers used for qPCR are listed in Table S1. The ΔΔCt method was used to normalize the levels of the target transcripts to that of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*).

2.4. Western blotting

Western blotting was performed as described previously [11]. Immunoreactive proteins were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol. The full-gel results of western blot analyses are shown in Fig. S1-S4.

2.5. Giemsa staining

Giemsa staining was performed as described previously [11].

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Gene expression data were log-transformed to provide an approximation of the normal distribution before analysis. Statistical analyses of gene expression were performed using the SAS statistical software (version 9.4; SAS Inst. Inc., Cary, NC). Data were analyzed using one-way or two-way analysis of variance (ANOVA). Comparisons between groups were performed using Dunnett's test or Tukey's test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

We first evaluated the effect of U0126, an MEK1/2 inhibitor [13], on myogenesis. The expression levels of *Myod* and *Myog* were increased upon treatment with U0126 for 4 days after differentiation stimulation (Fig. 1A). Treatment with U0126 at 5 μM or higher increased the expression of *Myod* and *Myog* by > 3-fold and > 10-fold, respectively. In contrast, treatment with U0126 (15 μM) decreased the expression of *Myf5*. *Mrf4* expression was not affected by U0126.

There are two types of muscles, namely fast-type and slow-type [14]. MYH1, MYH2, and MYL1 are highly expressed in fast-twitch muscles, whereas MYH7, MYL2, and MYL4 constitute slow-twitch type muscles [14]. MYL is divided into two classes: essential light chain and regulatory light chain. MYL1 is categorized as an essential light chain, whereas MYL2 and MYL4 are regulatory light chains [15].

The expression levels of *Myh1*, *Myh4*, and *Myh7* were increased by treatment with 5

μM U0126 (Fig. 1B). Treatment with 10 μM U0126 also increased *Myh7* expression. U0126 showed a potency to up-regulate *Myl1* and *Myl2* expression (Fig. 1C). In addition, although the mRNA levels of *Myl4* were increased, this increase was not statistically significant. Western blot analysis showed that MYH1/2 protein levels were increased upon treatment with U0126 at 5 μM or higher concentration (Fig. 1D, lane 1 *vs.* lanes 2-4), and that the level was the highest in cells treated with 5 μM U0126 (Fig. 1D, lane 2). Consistent with these results, Giemsa staining revealed the emergence of thick, long multinuclear cells after treatment with U0126 (Fig. 1E). These results suggest that endogenous ERK1/2 activity inhibits myogenesis, partly through the repression of *Myod* and *Myog* expression.

We next examined the effect of co-treatment with U0126 and vitamin C. *Myf5* mRNA levels were slightly decreased by U0126, whereas vitamin C had no effect on *Myf5* expression (Fig. 2A). U0126, but not vitamin C, increased the mRNA level of *Myod* (Fig. 2B). Treatment with AsAp, but not AsA alone, increased *Myog* expression slightly; U0126-induced *Myog* expression was enhanced by treatment with AsA or AsAp at 100 μM or 200 μM (Fig. 2C). Neither U0126 nor vitamin C affected the expression of *Mrf4* (Fig. 2D).

A marginal increase in *Myh1* expression was detected following the treatment with U0126 or vitamin C, as shown in the ANOVA table (Fig. 3A, *inlet*). U0126 increased the expression of *Myh4* and *Myh7*, and expression of *Myh4*, but not *Myh7* was increased by vitamin C (Fig. 3B, C). The expression levels of *Myl1* were increased by vitamin C in the absence of U0126, however, vitamin C did not affect its expression in cells treated with U0126 (Fig. 3D). The expression of *Myl2* and *Myl4* was increased by U0126; vitamin C had no effect on *Myl2* and *Myl4* mRNA levels, irrespective of the treatment with U0126 (Fig. 3E, F). Western blot analysis revealed that U0126-induced MYH1/2 expression was enhanced by AsA or AsAp treatment (Fig. 3G, lane

6 *vs.* lanes 7-10). Consistent with these results, thicker and longer multinucleated cells were evident after the co-treatment with U0126 and vitamin C (Fig. 3H).

Although vitamin C had no effect on the mRNA levels of *Myh1* in the presence of U0126 (Fig. 3A), MYH1/2 protein level was clearly increased by vitamin C in U0126-treated cells (Fig. 3D). The mRNA level of *Myh2* was close to the detection limit (data not shown). Thus, the data at the mRNA level were not always consistent with those at the protein level. Previous studies have also shown a mismatched relationship between *Myh*/MYH expression at the mRNA and protein levels [16, 17]. Dexamethasone decreased the protein levels of MYH without affecting the mRNA levels of *Myh*s in C2C12 cells, resulting from the induction of MuRF, an E3 ubiquitin ligase, and stimulation of MYH protein degradation [16]. Additionally, degradation of BCL-2 interacting cell death suppressors (BIS) led to a decrease in MYH protein levels, but the mRNA levels of *Myh*s were not affected [17]. In addition to its effect on *Myh1* mRNA levels, inhibition of endogenous ERK1/2 activity and vitamin C may stabilize MYH1/2 protein through post-transcriptional regulation, leading to inconsistent changes in the expression at mRNA and protein levels.

To evaluate the off-target effects of U0126 on MEK1/2 inhibition, we further evaluated the effect of PD98059, another MEK1/2 inhibitor [18]. Treatment with AsAp alone slightly increased the protein expression of MYH1/2 (Fig. 4A, lanes 1-3). Similar to U0126, PD98059 potentiated an increase in MYH1/2 protein level on day 4, and the expression level was enhanced by co-treatment with AsAp (Fig. 4A, lanes 1-3 *vs.* lanes 7-9). Consistent with these results, thick and long myotubes were efficiently formed upon co-treatment with PD98059 and AsAp (Fig. 4B), verifying the inhibitory role of endogenous ERK1/2 in myogenesis and the synergistic stimulation of myogenesis along with vitamin C by ERK1/2 inhibition.

As described above, the MAP kinases consist of three main families, namely ERK1/2, p38 MAP kinase, and JNK [4]. We also examined the role of the endogenous activity of p38 MAP kinase and JNK in myogenesis using SB203580 (an inhibitor for p38 MAP kinase [19]) and SP600125 (a JNK inhibitor [20]), respectively (Fig. 4). Although the treatment with SB203580 alone had no effect on MYH1/2 protein levels (Fig. 4A, lane 1 *vs.* lane 10), MYH1/2 expression was lower in cells treated with SB203580 and AsAp than in those treated with AsAp alone (Fig. 4A, lanes 2-3 *vs.* lanes 11-12). SB203580 alone did not induce morphological changes compared with vehicle treatment; however, compared with the treatment with AsAp alone, more mononuclear cells were detected when SB203580 and AsAp were used as a co-treatment (Fig. 4B), reflecting inhibition of myotube formation. These results suggest that endogenous p38 MAP kinase activity is required for myogenesis in the presence of vitamin C. Previously, treatment with SB203580 alone has been shown to repress the formation of myotubes [8]. The reasons for these discrepant results obtained for responses against SB203580 are currently unknown.

Treatment with SP600125 alone marginally increased the expression of MYH1/2 protein (Fig. 4A, lane 1 *vs.* lane 13), which was consistent with a previous study [21]. In addition, compared to the treatment with AsAp (200 μM) alone (Fig. 4A, lane 3), co-treatment with SP600125 and AsAp slightly increased MYH1/2 expression (Fig. 4A, lane 15). Thick and long myotubes were also efficiently formed by co-treatment with SP600125 and AsAp (Fig. 4B). These results suggest that the activity of endogenous JNK is similar to that of endogenous ERK, but is relatively weaker.

Previous studies have revealed the involvement of MAP kinase activity in vitamin C-mediated biological changes [22-24]; AsA induced cardiogenesis by inducing ERK phosphorylation and activation in white adipose tissue-derived stem cells [22]. Vitamin C was observed to reduce p38 MAP kinase phosphorylation during p53-

induced growth inhibition of human bladder cancer EJ cells [23]. Phosphorylation and activation of JNK were found to be associated with dehydroascorbic acid-induced steroidogenesis in human choriocarcinoma JAR cells [24]. Hence, we examined MAP kinase phosphorylation in C2C12 cells treated with the inhibitors and vitamin C to explore possible involvement of MAP kinase phosphorylation in the regulatory myogenesis (Fig. 4C). Treatment with U0126 or PD98059 expectedly decreased the phosphorylation of ERK (Fig. 4C, lanes1-3, 6-8, and 11-13). AsAp slightly increased ERK phosphorylation (Fig. 4C, lanes 1 and 11). Phosphorylation of JNK was increased by SB203580 treatment (Fig. 4C, lanes 1, 4, 6, 9, 11, and 14). JNK phosphorylation was decreased by treatment with U0126 but not PD98059 (Fig. 4C, lanes 1-3, 6-8, and 11-13). In summary, phosphorylated MAP kinase did not show any changes consistent with changes in myogenesis in C2C12 cells co-treated with a MEK1/2 inhibitor (U0126 and PD98059) and vitamin C.

The present study has revealed that: 1) endogenous ERK1/2 activity negatively regulates myogenesis; 2) vitamin C slightly promotes myogenesis; 3) inhibition of ERK activity greatly enhances vitamin C-stimulated myogenesis; 4) in contrast, inhibition of p38 MAP kinase represses myogenesis in the presence of vitamin C; and 5) the role of endogenous JNK activity is similar to that of endogenous ERK1/2 activity. Thus, myogenesis is modulated through the interaction of MAP kinase activity (an internal factor) and vitamin C (an external factor). As described above, we have previously shown that vitamin C-induced myotube formation was enhanced by the induction of mild ER stress prior to the differentiation stimulation of myogenesis (Diao et al., 2021). Collectively, these results suggest that vitamin C is a conditional factor that modulates myogenesis. The detailed mechanism by which endogenous MAP kinase activity regulates vitamin C-induced myogenesis need to be clarified in future studies.

Funding

This study was funded by Kyoto University. This funding source did not influence in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Acknowledgement

We would like to thank Editage (www.editage.com) for English language editing.

References

[1] N. Zanou, P. Gailly, Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways, Cell Mol. Life Sci. 70 (2013) 4117-4130. https://doi.org/10.1007/s00018-013-1330-4.

[2] J. Chal, O. Pourquié, Making muscle: skeletal myogenesis in vivo and in vitro, Development 144 (2017) 2104-2122. https://doi.org/10.1242/dev.151035.

[3] W. Chen, D. Datzkiw, M.A. Rudnicki, Satellite cells in ageing: use it or lose it, Open Biol. 10 (2020) 200048. https://doi.org/10.1098/rsob.200048.

[4] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (2002) 9-18. https://doi.org/10.1038/sj.cr.7290105.

[5] C. Jo, B.G. Jang, S.A. Jo, MEK1 plays contrary stage-specific roles in skeletal myogenic differentiation, Cell. Signal. 21 (2009) 1910-1917. https://doi.org/10.1016/j.cellsig.2009.08.008.

[6] J. Li, S.E. Johnson, ERK2 is required for efficient terminal differentiation of skeletal myoblasts, Biochem. Biophys. Res. Commun. 345 (2006) 1425-1433. https://doi.org/10.1016/j.bbrc.2006.05.051.

[7] Y. Feng, L.L. Niu, W. Wei, W.Y. Zhang, X.Y. Li, J.H. Cao, S.H. Zhao, A feedback circuit between miR-133 and the ERK1/2 pathway involving an exquisite mechanism for regulating myoblast proliferation and differentiation, Cell Death Dis. 4 (2013) e934. https://doi.org/10.1038/cddis.2013.462.

[8] Z. Wu, P.J. Woodring, K.S. Bhakta, K. Tamura, F. Wen, J.R. Feramisco, M. Karin, J.Y. Wang, P.L. Puri, p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps, Mol. Cell. Biol. 20 (2000) 3951-3964. https://doi.org/10.1128/mcb.20.11.3951-3964.2000.

[9] Y. Mitsumoto, Z. Liu, A. Klip, A long-lasting vitamin C derivative, ascorbic acid

2-phosphate, increases myogenin gene expression and promotes differentiation in L6 muscle cells, Biochem. Biophys. Res. Commun. 199 (1994) 394-402. https://doi.org/10.1006/bbrc.1994.1242.

[10] A. Shima, J. Pham, E. Blanco, E.R. Barton, H.L. Sweeney, R. Matsuda, IGF-I and vitamin C promote myogenic differentiation of mouse and human skeletal muscle cells at low temperatures, Exp. Cell Res. 317 (2011) 356-366. https://doi.org/10.1016/j.yexcr.2010.11.001.

[11] Z. Diao, T. Matsui, M. Funaba, Stimulation of myogenesis by ascorbic acid and capsaicin, Biochem. Biophys. Res. Commun. 568 (2021) 83-88. https://doi.org/10.1016/j.bbrc.2021.06.067.

[12] R. Kida, H. Yoshida, M. Murakami, M. Shirai, O. Hashimoto, T. Kawada, T. Matsui, M. Funaba, Direct action of capsaicin in brown adipogenesis and activation of brown adipocytes, Cell. Biochem. Funct. 34 (2016) 34-41. https://doi.org/10.1002/cbf.3162.

[13] M.F. Favata, K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feeser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, R.A. Copeland, R.L. Magolda, P.A. Scherle, J.M. Trzaskos, Identification of a novel inhibitor of mitogen-activated protein kinase kinase, J. Biol. Chem. 273 (1998) 18623-18632. https://doi.org/10.1074/jbc.273.29.18623.

[14] S. Schiaffino, A.C. Rossi, V. Smerdu, L.A. Leinwand, C. Reggiani, Developmental myosins: expression patterns and functional significance, Skelet. Muscle 5 (2015) 22. https://doi.org/10.1186/s13395-015-0046-6.

[15] Y.H. Sitbon, S. Yadav, K. Kazmierczak, D. Szczesna-Cordary, Insights into myosin regulatory and essential light chains: a focus on their roles in cardiac and skeletal muscle function, development and disease, J. Muscle Res. Cell Motil. 41 (2020) 313-327. https://doi.org/10.1007/s10974-019-09517-x.

[16] B.A. Clarke, D. Drujan, M.S. Willis, L.O. Murphy, R.A. Corpina, E. Burova, S.V. Rakhilin, T.N. Stitt, C. Patterson, E. Latres, D.J. Glass, The E3 ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle, Cell Metab. 6 (2007) 376-385. https://doi.org/10.1016/j.cmet.2007.09.009.

[17] J. Hong, J.S. Park, H. Lee, J. Jeong, H. Hyeon Yun, H. Yun Kim, Y.G. Ko, J.H. Lee, Myosin heavy chain is stabilized by BCL-2 interacting cell death suppressor (BIS) in skeletal muscle, Exp. Mol. Med. 48 (2016) e225. https://doi.org/10.1038/emm.2016.2.

[18] D.T. Dudley, L. Pang, S.J. Decker, A.J. Bridges, A.R. Saltiel, A synthetic inhibitor of the mitogen-activated protein kinase cascade, Proc. Natl. Acad. Sci. USA 92 (1995) 7686-7689. https://doi.org/10.1073/pnas.92.17.7686.

[19] A. Cuenda, J. Rouse, Y.N. Doza, R. Meier, P. Cohen, T.F. Gallagher, P.R. Young,

J.C. Lee, SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1, FEBS Lett. 364 (1995) 229-233. https://doi.org/10.1016/0014-5793(95)00357-f.

[20] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, Proc. Natl. Acad. Sci. USA 98 (2001) 13681-13686. https://doi.org/10.1073/pnas.251194298.

[21] S.J. Xie, J.H. Li, H.F. Chen, Y.Y. Tan, S.R. Liu, Y. Zhang, H. Xu, J.H. Yang, S. Liu, L.L. Zheng, M.B. Huang, Y.H. Guo, Q. Zhang, H. Zhou, L.H. Qu, Inhibition of the JNK/MAPK signaling pathway by myogenesis-associated miRNAs is required for skeletal muscle development, Cell Death Differ. 25 (2018) 1581-1597. https://doi.org/10.1038/s41418-018-0063-1.

[22] W. Liu, H. Liu, Y. Wang, L. Zhang, C. Wang, H. Li, Ascorbic acid induces cardiac differentiation of white adipose tissue-derived stem cells, Mol. Cell. Biochem. 450 (2019) 65-73. https://doi.org/10.1007/s11010-018-3373-1.

[23] J.E. Kim, D.H. Jin, S.D. Lee, S.W. Hong, J.S. Shin, S.K. Lee, D.J. Jung, J.S. Kang, W.J. Lee, Vitamin C inhibits p53-induced replicative senescence through suppression of ROS production and p38 MAPK activity, Int. J. Mol. Med. 22 (2008) 651-655. https://doi.org/10.3892/ijmm_00000068.

[24] Y. Wang, C. Tang, M. Wu, Y. Pan, H. Ruan, L. Chen, H. Yao, H. Zhu, X. Wu, Dehydroascorbic acid taken up by glucose transporters stimulates estradiol production through inhibition of JNK/c-Jun/AP1 signaling in JAR cells, Mol. Hum. Reprod. 20 (2014) 799-809. https://doi.org/10.1093/molehr/gau036.

Figure legends

Figure 1. Enhancement of myogenesis by U0126 in C2C12 cells

One day after reaching the confluence (day 0), C2C12 cells were treated with different concentrations (0, 5, 10, or 15 μM) of U0126 in the differentiation medium for 4 days. The expression levels of *Mrf*s (A), *Myh*s (B), and *Myl*s (C) were quantified by RT-qPCR. The expression levels of the respective genes in the control cells were set to 1. Data are presented as mean ± SEM (n = 3). **: $P < 0.01$ *vs.* control cell. (D) MYH1/2 protein level was examined using western blot analysis. The expression level of β-actin was used as the reference. (E) Representative image of Giemsa-stained C2C12 cells on day 4. Scale bar, 50 μM.

Figure 2. Modulation of expression levels of *Mrf*s by U0126 and vitamin C

One day after reaching the confluence (day 0), C2C12 cells were cultured in differentiation medium with vitamin C (AsA or AsAp at 100 μM or 200 μM) and U0126 (10 μM) for 4 days. The expression levels of *Myf5* (A), *Myod* (B), *Myog* (C), and *Mrf4* (D) were quantified by RT-qPCR. The expression levels of the respective genes in the control cells were set to 1. Data are presented as mean ± SEM (n = 3). The results of ANOVA are expressed as an *inlet*; U: U0126, VC: vitamin C, U × VC: interaction of U0126 and vitamin C. a-d: Mean values that do not have a common letter on the bar differ significantly ($P < 0.05$).

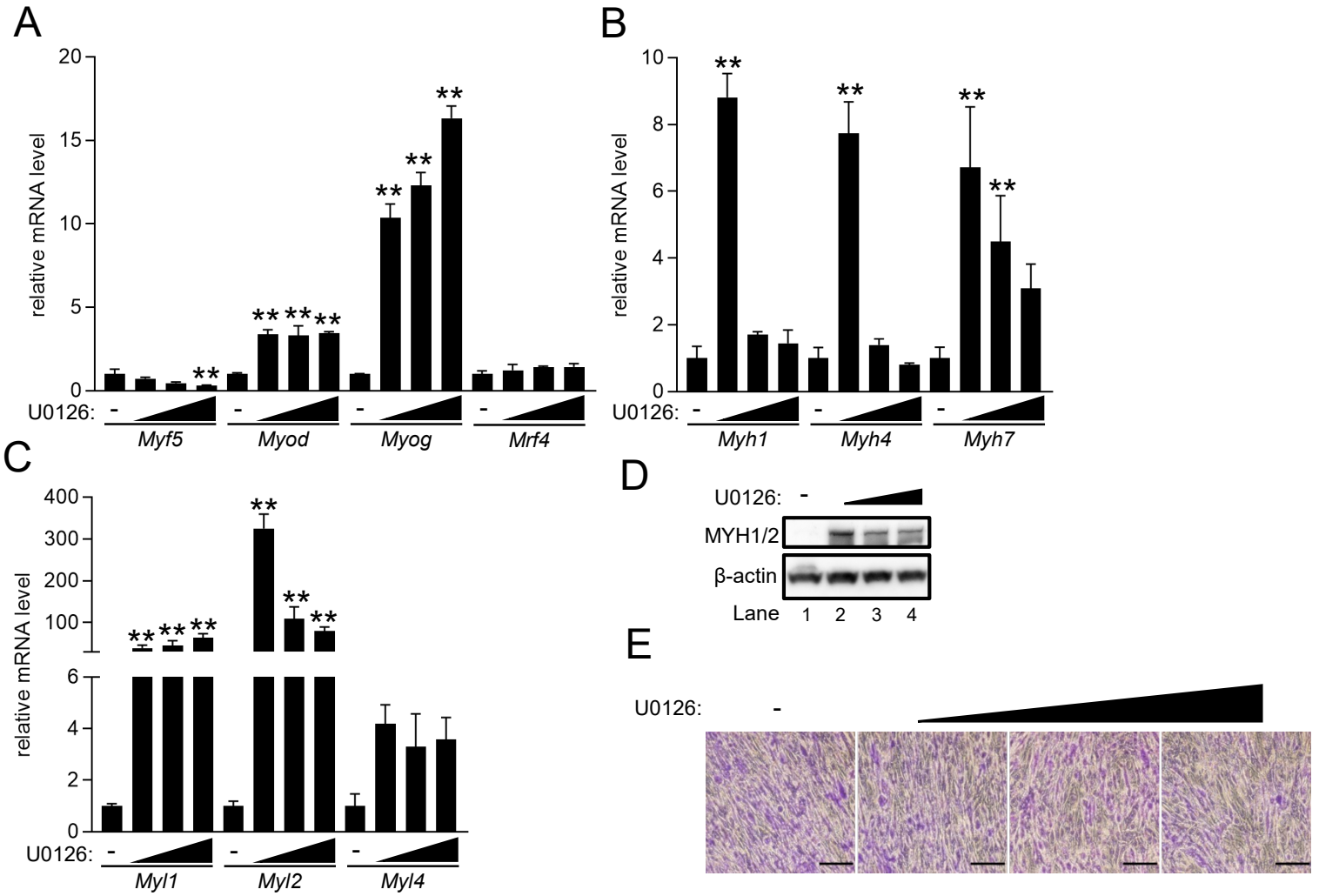
Figure 3. Further enhancement of U0126-induced myogenesis by vitamin C

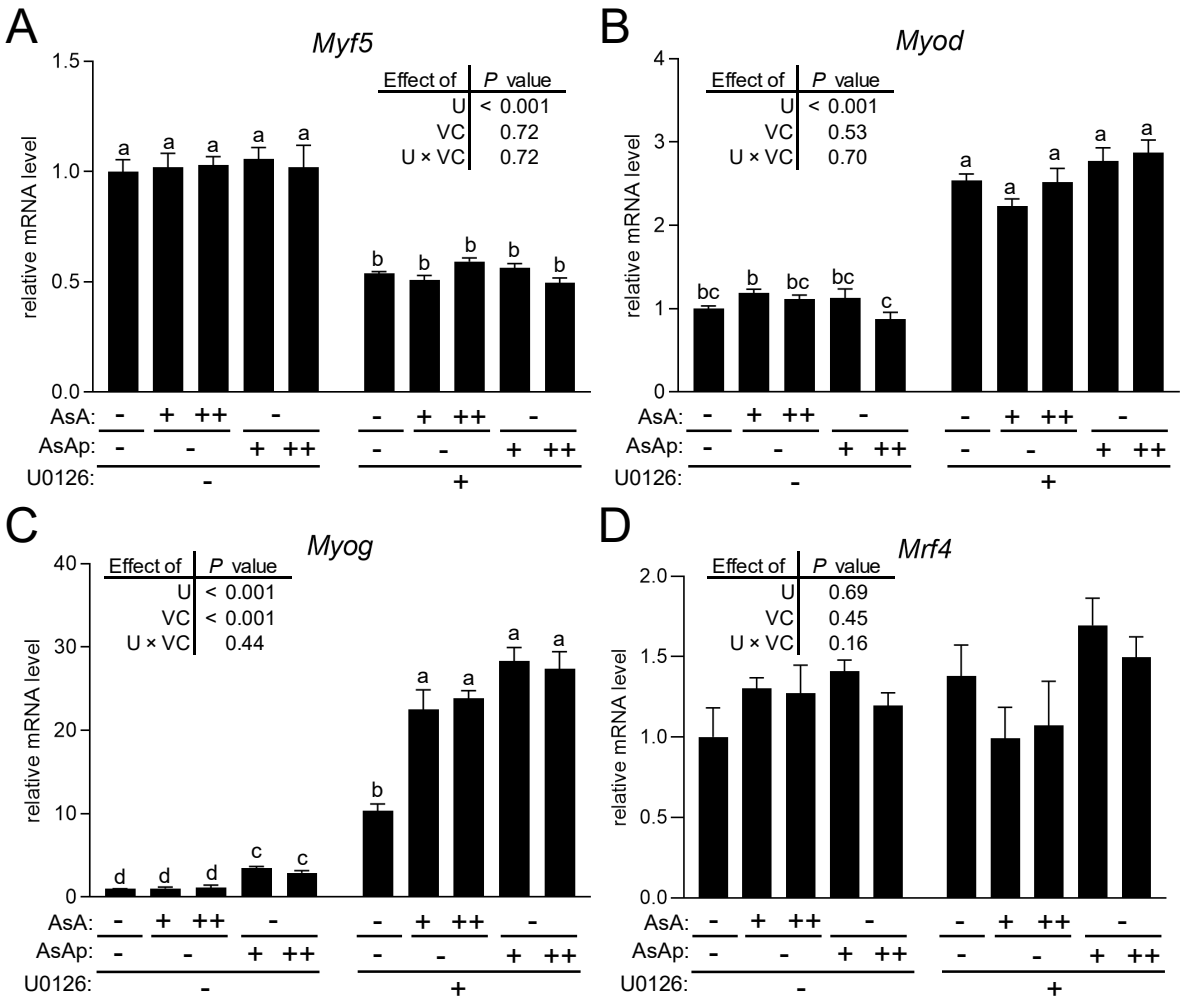
One day after reaching the confluence (day 0), C2C12 cells were cultured in differentiation medium with vitamin C (AsA or AsAp at 100 μM or 200 μM) and U0126 (10 μM) for 4 days. The expression levels of *Myh1* (A), *Myh4* (B), *Myh7* (C), *Myl1* (D), *Myl2* (E), and *Myl4* (F) were quantified by RT-qPCR. The expression levels of the respective genes in the control cells were set to 1. Data are presented as mean ± SEM (n = 3). The results of ANOVA are expressed as an *inlet*; U: U0126, VC: vitamin

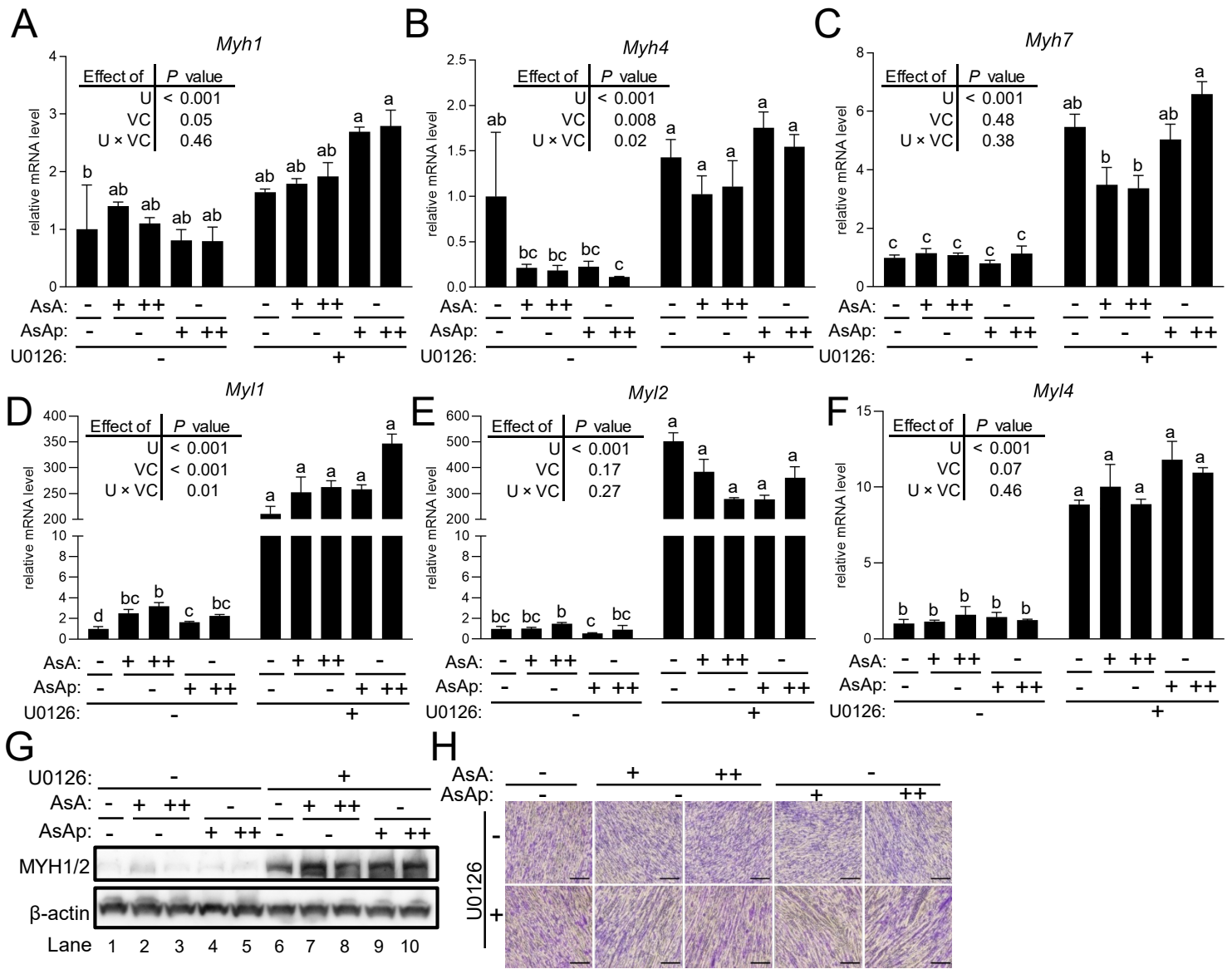
C, U × VC: interaction of U0126 and vitamin C. a-c: Mean values that do not have a common letter on the bar differ significantly ($P < 0.05$). (G) MYH1/2 protein level was examined using western blot analysis. The expression level of β-actin was used as the reference. (H) Representative image of Giemsa-stained C2C12 cells on day 4. Scale bar, 50 μM.

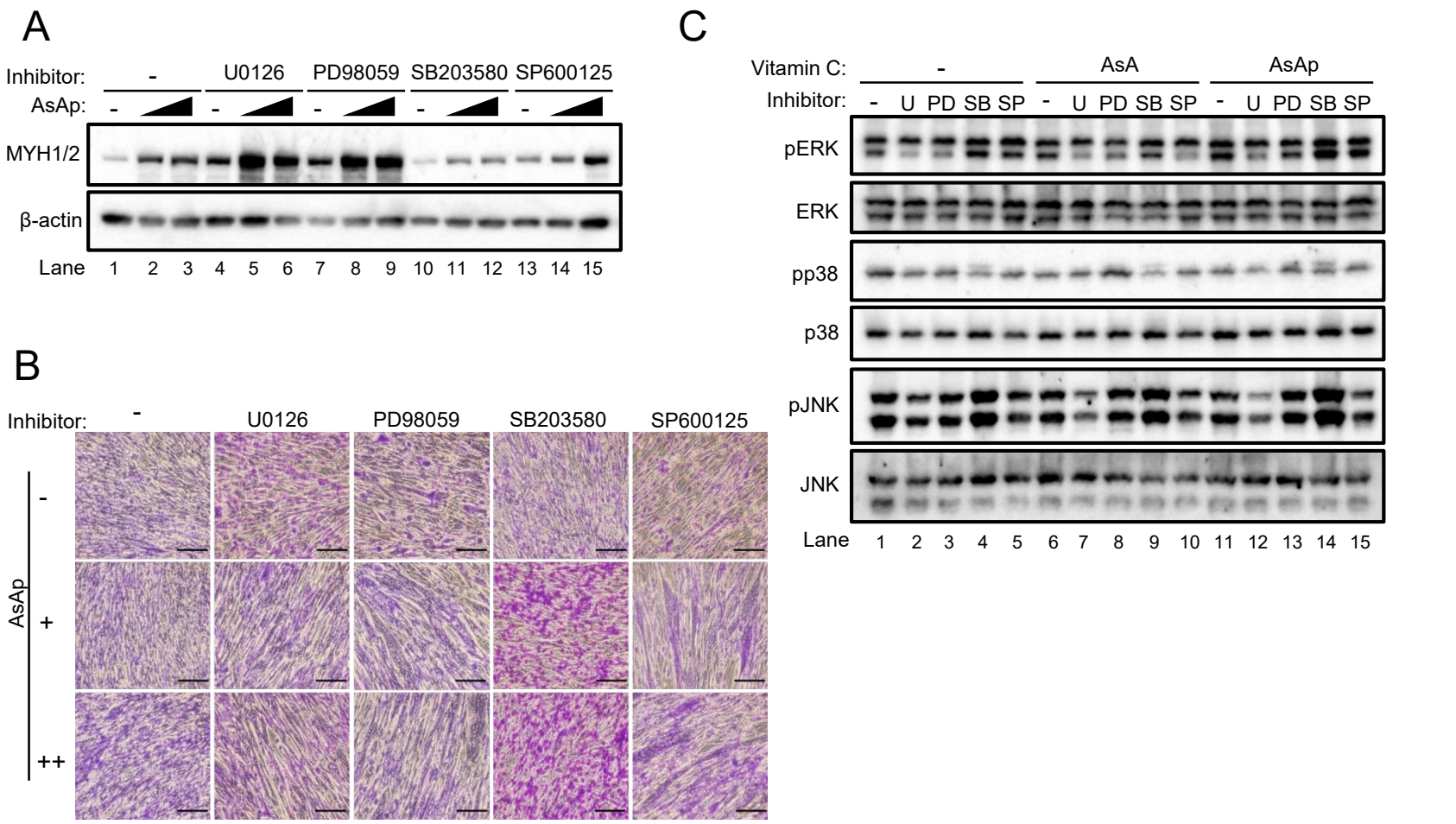
Figure 4. Modulation of myogenesis by MAP kinase inhibitors and vitamin C

One day after reaching the confluence (day 0), C2C12 cells were cultured in differentiation medium with vitamin C (AsAp at 100 μM or 200 μM (A, B) or AsA or AsAp at 200 μM (C)) and the indicated MAP kinase inhibitors (U0126 (10 μM), PD98059 (20 μM), SB203580 (10 μM), or SP600125 (10 μM)) for 4 days (A, B) or 1 h (C). MYH1/2 protein level (A) or phosphorylation level of MAP kinases (C) were examined by western blot analysis. The expression level of β-actin was used as the reference. (B) Representative image of Giemsa-stained C2C12 cells on day 4. Scale bar, 50 μM.


A
relative mRNA level
20
15
10
5
0
U0126: - - - -
Myf5
Myod
Myog
Mrf4
B
relative mRNA level
10
8
6
4
2
0
U0126: - - -
Myh1
Myh4
Myh7
C
relative mRNA level
400
300
200
100
6
4
2
0
U0126: - - -
Myl1
Myl2
Myl4
D
U0126: -
MYH1/2
β-actin
Lane 1 2 3 4
E
U0126: -


A
Myf5
relative mRNA level
1.5
1.0
0.5
0.0
Effect of | P value
U | < 0.001
VC | 0.72
U × VC | 0.72
a a a a a
b b b b b
AsA: - + ++ - - + ++ -
AsAp: - - + ++ - - + ++
U0126: - +
B
Myod
relative mRNA level
4
3
2
1
0
Effect of | P value
U | < 0.001
VC | 0.53
U × VC | 0.70
bc b bc bc c
a a a a a
AsA: - + ++ - - + ++ -
AsAp: - - + ++ - - + ++
U0126: - +
C
Myog
relative mRNA level
40
30
20
10
0
Effect of | P value
U | < 0.001
VC | < 0.001
U × VC | 0.44
d d d c c
b a a a a
AsA: - + ++ - - + ++ -
AsAp: - - + ++ - - + ++
U0126: - +
D
Mrf4
relative mRNA level
2.0
1.5
1.0
0.5
0.0
Effect of | P value
U | 0.69
VC | 0.45
U × VC | 0.16
AsA: - + ++ - - + ++ -
AsAp: - - + ++ - - + ++
U0126: - +


A
Myh1
B
Myh4
C
Myh7
D
Myl1
E
Myl2
F
Myl4
relative mRNA level
Effect of
P value
U
VC
U × VC
AsA:
AsAp:
U0126:
G
U0126:
AsA:
AsAp:
MYH1/2
β-actin
Lane 1 2 3 4 5 6 7 8 9 10
H
AsA:
AsAp:
U0126


A
Inhibitor: - U0126 PD98059 SB203580 SP600125
AsAp: - - - - -
MYH1/2
β-actin
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Inhibitor: - U0126 PD98059 SB203580 SP600125
AsAp
-
+
++
C
Vitamin C: - AsA AsAp
Inhibitor: - U PD SB SP - U PD SB SP - U PD SB SP
pERK
ERK
pp38
p38
pJNK
JNK
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Supplementary information

Enhancement of vitamin C-induced myogenesis by inhibition of extracellular signal-regulated kinase (ERK) 1/2 pathway

Xiajie Fu, Tohru Matsui, and Masayuki Funaba*

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

*Corresponding author: Masayuki Funaba, Ph.D.
Division of Applied Biosciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan
Tel.: +81-75-753-6055
Fax: +81-75-753-6344
E-mail: funaba.masayuki.8w@kyoto-u.ac.jp

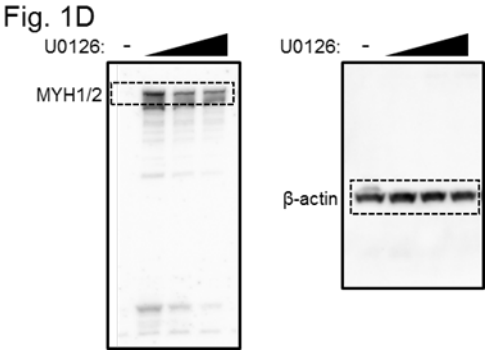


Figure S1. Images of full-length blot shown in Fig. 1D

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 1D.

Fig. 3G

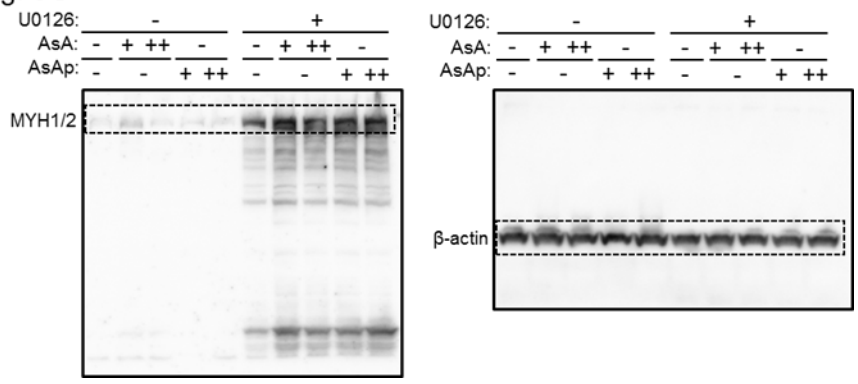


Figure S2. Images of full-length blot shown in Fig. 3G

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 3G.

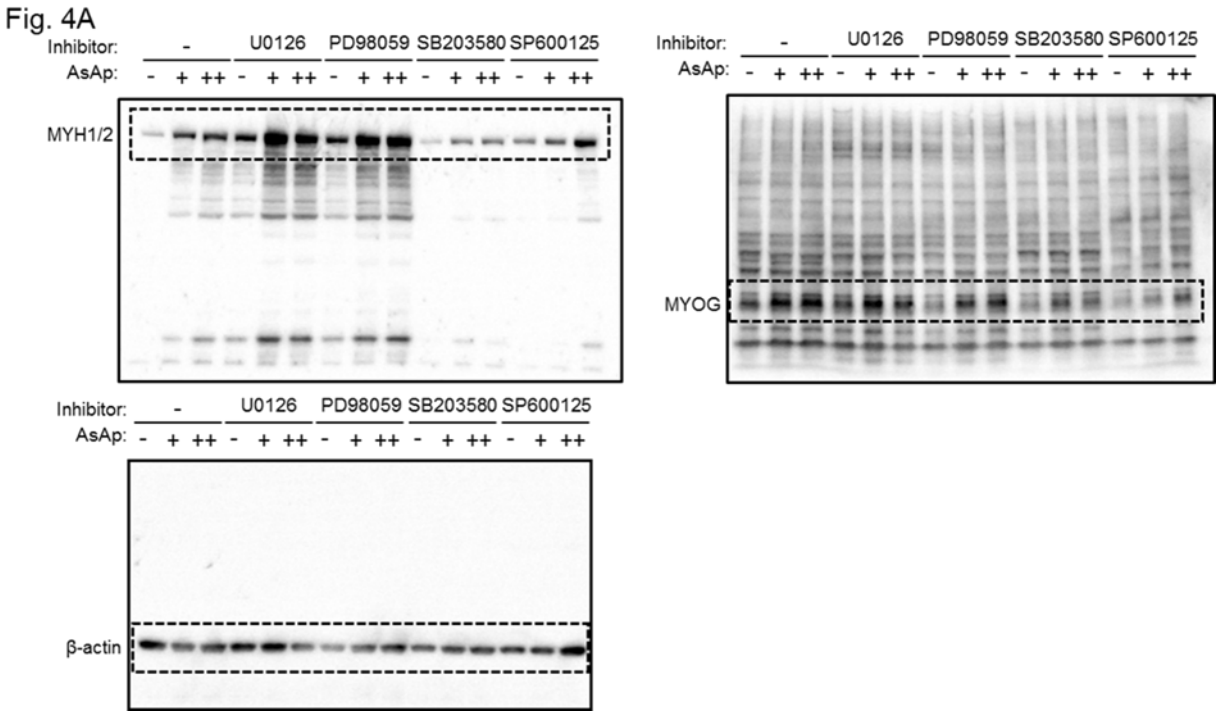


Figure S3. Images of full-length blot shown in Fig. 4A

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 4A.

Fig. 4C

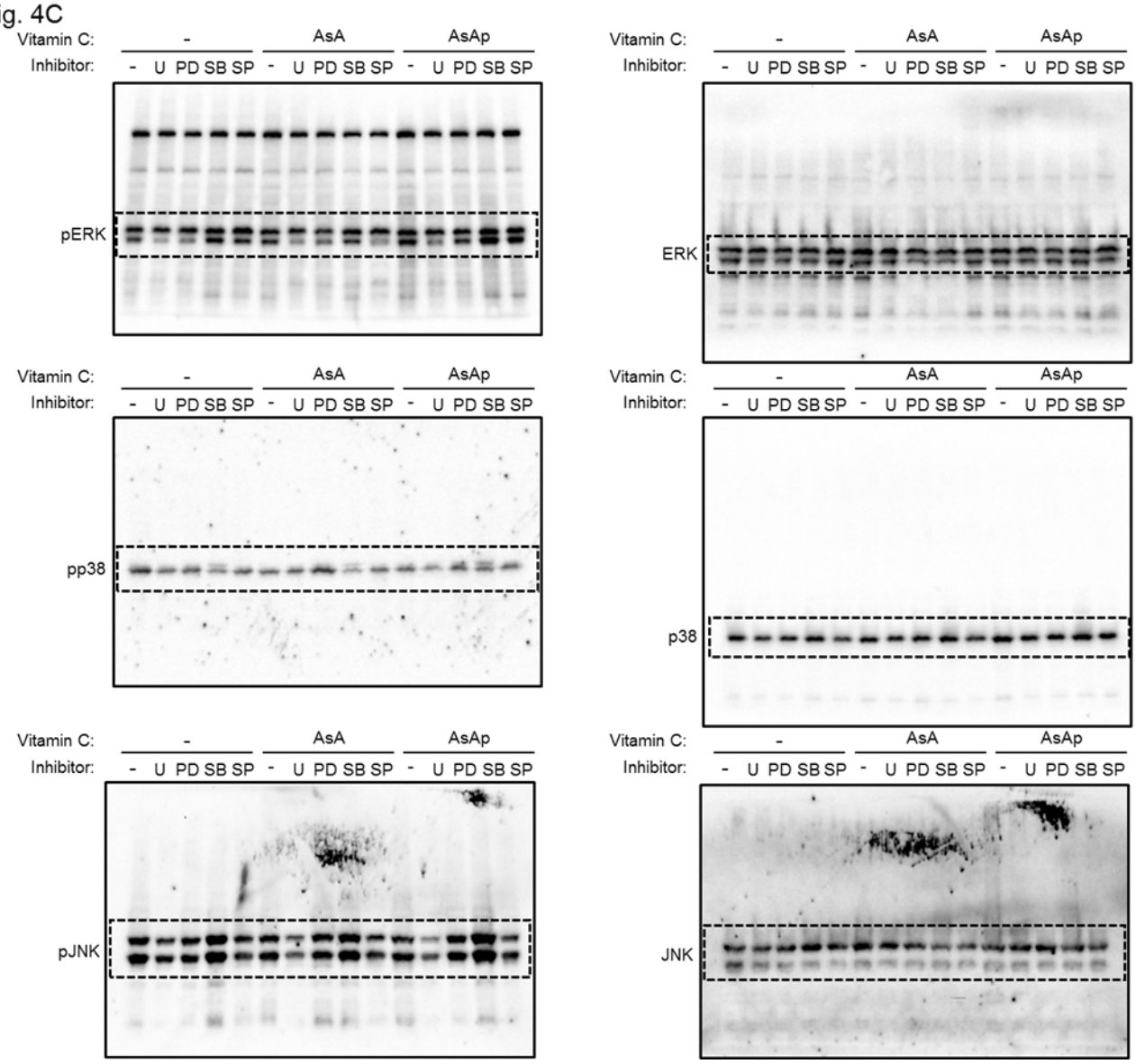


Figure S4. Images of full-length blot shown in Fig. 4C

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 4C.

Table S1. Oligonucleotide PCR primers for RT-qPCR

	5’-primer	3’-primer
Myogenic regulatory factors		
Mrf4	5’-CAGCTACAAACCCAAGCAAG-3’	5’-AGGCATCCACGTTTGCTC-3’
Myod	5’-AGCACTACAGTGGCGACTCA-3’	5’-GGCCGCTGTAATCCATCAT-3’
Myog	5’-ACAGGCCTTGCTCAGCTC-3’	5’-CGCTGTGGGAGTTGCATT-3’
Myf5	5’-CTGCTCTGAGCCCACCAG-3’	5’-GACAGGGCTGTTACATTCAGG-3’
Myosin heavy chains		
Myh1	5’-GGACCCACGGTCGAAGTTG-3’	5’-GGCTGCGGGCTATTGGTT-3’
Myh4	5’-CCGAGCAAGAGCTACTGGA-3’	5’-TGTTGATGAGGCTGGTGTTC-3’
Myh7	5’-CATCCCCAATGAGACAAAGTC-3’	5’-CGGAAGTCCCCATAGAGAATG-3’
Myosin light chains		
Myl1	5’-GGATTGGAGCTGCCTTCAGT-3’	5’-GCCTCCTTGAAGTCGGCAAT-3’
Myl2	5’-CCTCAGACACCATGGCACC-3’	5’-GTCGATGAAGCCGTCTCTGT-3’
Myl4	5’-GACAGTGTCCATATACCTGCCTG-3’	5’-TCAGCACTGAAGTCTATCTTCACAC-3’
Reference		
Hprt1	5’-GTTATGACCTAGATTTGTTTTGTA-3’	5’-CTTCATGACATCTCGAGCAAGTCT-3’