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Research Article

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Induction of Myogenic Differentiation in Myoblasts by Electrical Stimulation

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| Abstract |

PURPOSE: While electrical stimulation (ES) is known to be a safe and flexible tool in rehabilitation therapy, it has had limited adoption in muscle regeneration. This study was performed to investigate whether ES can induce myogenic differentiation and to clarify the mechanism underlying the effects of ES on myogenic differentiation.

METHODS: This study used rat L6 cell lines as myoblasts for myogenic differentiation. Electric stimulation was applied to the cells using a C-Pace EP culture pacer (IonOptix, Westwood, Ma, USA). The gene expressions of myogenic markers were examined using qPCR and immunochemistry.

RESULTS: Our study showed that ES increased the thickness and length of myotubes during myogenic differentiation. It was found that ES increased the expression of myogenic markers, such as MyoD and Myogenin, and also activated the fusion of the myoblast cells. In addition, ES suppressed the expression of small GTPases, which can explain why ES promotes myogenic differentiation.

CONCLUSION: We found that ES induced myogenic differentiation by suppressing small GTPases, inhibiting cell division. We suggest that ES-based therapies can contribute to the development of safe and efficient muscle regeneration.

Key Words: Electrical stimulations, Myoblast, Myogenesis, Small GTPase

Ⅰ. Introduction

With gradual increase in lifespan, increased number of elderly people are suffering from sarcopenia or muscle dystrophy. These muscle disorders can be major causes of brain disease or diabetes, as well as decreased motor function [1-3]. Muscle tissues are often damaged by tissue loss due to congenital defects, large traumas, tumors removal, sarcopenia, and muscle atrophy. Although autologous or allogenic stem cell-based cell therapies have been attempted for repairing damaged muscle tissues, these have some limitations, such as the low efficiency of muscle regeneration, survival rate of the source cells at the injured site, and immune rejection response [4]. Therefore, it would be necessary to develop a novel technique for inducing myogenic differentiation while providing high efficiency and high safety for muscle regeneration [5].

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Since previous studies have identified growth factors and cytokines involved in myogenic differentiation, therapies based on the biochemical factors and genetic manipulation for inducing myogenic differentiation have been developed; however, many limitations have been identified with respect to their clinical application. These limitations include high time consumption, high cost, and high risks, such as increased risk of teratomas formation [6,7]. Recent studies have shown that physical factors can influence many cellular functions [8,9]. It has been established that an electrical reaction is generated during physiological activities in various tissues within the body and is associated with various pathological phenomena [10,11]. Previous studies showed that electrical stimulation induces cellular responses through voltage-dependent channels, ionic channels, signaling by gap junctions, changes in ion distribution in the extracellular matrix, and activation of cell secretion [12-14]. These results implicate that electrical stimulations can influence cell differentiation by modulating various signal transductions. Previous studies have used electrical stimulation for inducing muscle contraction after myotube formation [15,16]. Although some studies reported that electrical stimulation has positive effects on myogenic differentiation [17], they were unable to clarify the underlying mechanism behind the effects of electrical stimulation on myogenic differentiation. Therefore, in the present study, we investigated whether electrical stimulation can promote myogenic differentiation and evaluated the possible mechanism underlying the effects of electrical stimulation on myogenic differentiation.

Ⅱ. Methods

1. Cell culture

Rat L6 myoblast cells were maintained in Dulbecos's modified Eagle's medium (DMEM; Welgene, Seoul Korea), supplemented with 10% fetal bovine serum (FBS; RMBIO, USA) (Growth medium) and 1% antibiotic-antimycotic (Anti-anti; Gibco, USA) at 37°C in a humidified atmosphere of 5% CO_2. L6 myoblast cells were seeded in 35 mm dishes at the density of 3×10^5 cells/mL for differentiation into myotube. After 48 hours of incubation, the culture media were replaced by DMEM, which included 2% FBS (Differentiation medium) and 1% Anti-anti; then, the differentiating medium was replaced every 2 days.

2. Electrical stimulation (ES)

ES was applied to L6 Rat skeletal myoblast cells using a C-Pace EP culture pacer (IonOptix, Westwood, Ma, USA), which is a multi-channel stimulator designed for chronic stimulation of bulk quantities of the cultured cells. This device contacts the carbon electrodes in the 35 mm dish (ThermoFisher Scientific, USA) and emits bipolar pulses. ES - with an electrical field of 1 V/cm, duration of 5 ms, and frequency of 1 Hz - was continuously applied to the L6 cells after the cells reached 80%~90% of confluency for 7 days. After ES treatment, cell lysis was performed with Trizol solution (Invitrogen) for gene expression analysis.

3. qPCR analysis

Total RNA was isolated from various L6 Rat skeletal muscle cell line cultures using the Direct-zol™ RNA MiniPrep (Zymo Research Corporation, Irvine, CA, U.S.A.) in accordance with the manufacturer's protocol. RNA concentrations were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and reverse transcription reactions were performed using .2 μg of total RNA with a TOPscript cDNA synthesis kit (enzynomics, Daejeon, Korea). The real-time PCRs for MyoD (Myogenic Differentiation 1), myogenin (myogenic factor 4), ECT2 (Epithelial Cell Transforming 2), DOCK1 (Dedicator of cytokinesis protein 1), LRRK2 (Leucine-rich repeat kinase 2), and VAV2 (Vav guanine nucleotide exchange factor 2) were performed

Table 1. The Primer Sequences for qPCR Analysis of Myogenic Markers and Small GTPases

Gene	Forward primers (5`-3`)	Reverse primers (5`-3`)
MyoD1	GCGACAGCCGATGACTTCTAT	GGTCCAGGTCCTCAAAAAAGC
Myogenin	GACCCTACAGGTGCCCACAA	ACATATCCTCCACCGTGATGCT
ECT2	AGTTCCTCCAAAGCAGTCGG	TCCTCGGGTGCAAGAATAGG
DOCK1	CTGCCGAAGAAGATGCACTC	AGGATCACAGCCTCAGACTG
LRRK2	TGTCATCTCCACCATCCCAG	CAATGCGATTCTGTGCTGGA
VAV2	CTGCAAGACGGAAGACATGA	ATCTTGCAGGCTTTGCAGTT
GAPDH	CCACCAACTGCTTAGCACC	GCCAAATTCGTTGTCATACC

using TOPreal qPCR 2X Premix SYBR Green (enzynomics, Daejeon, Korea). Primer sequences are described in Table 1. Real-time PCRs were performed with a StepOnePlus™ instrument (Applied Biosystems, Grand Island, NY, USA) at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 sec, extension at 60°C for 15 sec, and annealing at 72°C for 15 sec. Gene expression levels were normalized to that of GAPDH Housekeeping gene, and relative gene expression was computed using the ddCT method.

4. Immunofluorescence staining

The L6 myoblast cells were washed twice in phosphate buffered saline (PBS) and were fixed in 4% paraformaldehyde for 20 min at room temperature. Thereafter, the cells were washed three times using PBS. After blocking in PBS containing 5% goat serum and .3% Triton × 100 for 40 min at room temperature, the cells were incubated with rabbit anti-MHC (MF 20) antibody (1:10; DSHB, USA) at 4°C overnight, washed three times in PBS containing .1% Triton × 100, and then, incubated with Alexa594 conjugated secondary antibody (1:200; Invitrogen) for 60 min at room temperature in the dark. Subsequently, the cells were washed three times in PBS, containing .1% Triton × 100, and nuclei were stained with Hoechst 33258 (Dojindo, Tokyo, Japan). Finally, the cells were incubated for 5 minutes at room temperature and washed 3 times with PBS containing .1% Triton×100. The stained cells were visualized using a fluorescence microscopy (Olympus, Tokyo, Japan).

5. Fusion Index

The cells stained with MHC (Red) and Hoechst 33258 (Blue) were used. Three images were taken at random, using fluorescence microscopy (Olympus, Tokyo, Japan). Next, the total number of nuclei and the number of nuclei inside myotubes were calculated. The fusion index was calculated as the number of nuclei in myotubes divided by the total number of nuclei.

$$\text{Fusion Index } (\%) = 100 \times \frac{\text{Number of the nuclei inside Myotubes}}{\text{Total number of nuclei}}$$

6. Statistical analyses

SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) was used to conduct all statistical analyses. All values were expressed as the mean±SD. Comparisons between the two mean values were evaluated using Paired t-test, and comparisons between three or more groups **Ed note: values?** were evaluated using one-way ANOVA, followed by LSD post-hoc analysis. Statistical significance was accepted at p-value<.05.

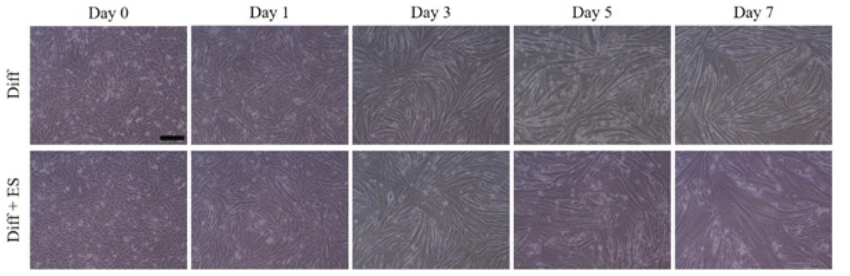

Fig. 1. Morphological observation of L6 differentiation, with or without electrical stimulation. L6 cells were cultured in the differentiation medium without electrical stimulation (Diff) or with electrical stimulation (Diff+ES). Scale bars, 100 μm

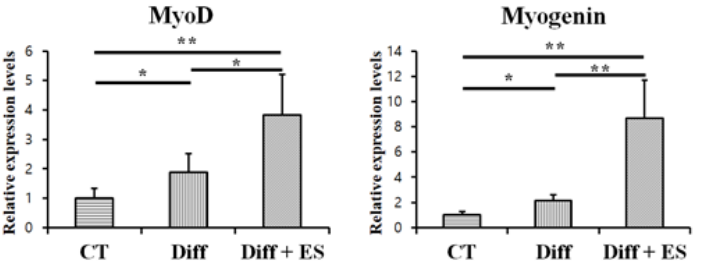

Fig. 2. Electrical stimulation enhances mRNA expression of myogenesis markers and MHC ratio in L6 cells. (A) qPCR analysis of myogenic markers, MyoD, and Myogenin in L6 cells after culture for 7 days in the maintenance medium (CT), differentiation medium (DM), and differentiation medium with ES (ES). Data are presented as the means±standard deviations (S.D.). Statistical analyses were performed using ANOVA (LSD test); **p<.01, *p<.05

Ⅲ. RESULTS

1. Electrical stimulation promotes myotube formation

The electric stimulation device allows carbon electrodes to be immersed in a culture medium containing the cells, and then the electricity can be flowed at an intensity of 1 v / cm, 5 ms and 1 Hz. After the L6 myoblast cells were cultured to reach a confluency of about 80 to 90%, the cells were cultured in the differentiation medium without electrical stimulation (DM) or with electric stimulation (ES). We found that the L6 cells treated with ES formed much thicker and larger myotubes than the non-treated L6 cells (Fig. 1). These results indicate that ES promotes myogenic differentiation.

2. Electrical stimulation increases myogenic transcription factors

We then examined whether ES increases the expression levels of myogenic markers, such as MyoD and Myogenin. Thus, we performed qPCR analyses for analyzing the mRNA levels of the myogenic markers. It was shown that the differentiation medium (Diff) significantly increased gene expression of the myogenic markers, such as MyoD and myogenin on day 7, while ES significantly enhanced the gene expression of the myogenic markers, revealing a 2-fold increase in MyoD and a 4.5-fold increase in myogenin (Fig. 2). This suggests that ES more efficiently accelerates myogenic differentiation of myoblast cells (Fig. 2).

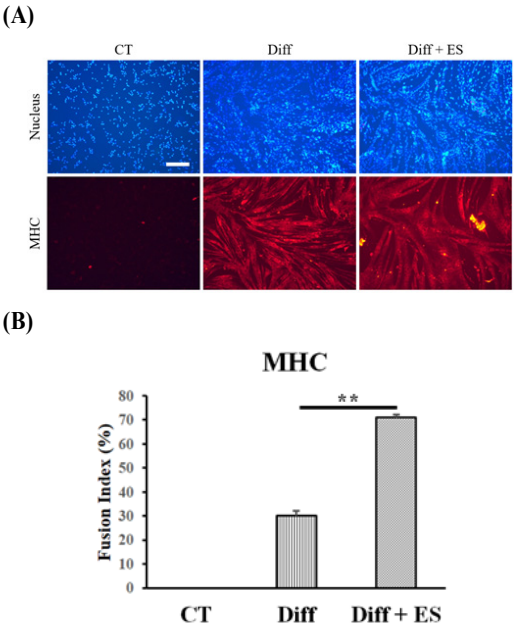

Fig. 3. (B) Immunofluorescent staining (Nuclei; blue) and myosin heavy chain (MHC; Red). Scale bars, 500 μm. (C) Fusion index analysis of immunofluorescent stained L6 cells. Fusion index was calculated as the number of nuclei in myotubes divided by the total number of nuclei. Statistical analyses were performed using paired t-test; **p<.01, *p<.05

3. Electrical stimulation induces fusion of myoblast cells during myogenic differentiation

Immunostaining analysis showed that myotubes formed in the differentiation medium without ES or with ES expressed myosin heavy chain (MHC) strongly,whereas myoblast cells in the maintenance medium did not show strong expression (Fig. 3A). Moreover, the cells cultured in the differentiation medium with ES treatment showed a 2.5-fold higher myotube fusion than those cultured in the differentiation medium without ES (Fig. 3B). This indicates that ES promotes myogenic differentiation by activating the fusion processes.

4. Electrical stimulation suppressed small GTPase signaling during myotube formation

We examined whether ES influences the expression level of small GTPase, such as ECT2 (Epithelial Cell Transforming 2), DOCK1 (Dedicator of cytokinesis protein 1), LRRK2 (Leucine-rich repeat kinase 2), and VAV2 (Vav guanine nucleotide exchange factor 2). qPCR analyses showed that the expression levels of small GTPases in the cells cultured in the differentiation medium were lower than those in the cells cultured in the maintenance medium. Furthermore, it was found that ES more strongly suppressed the expression levels of small GTPases (Fig. 4). This implicates that ES-induced myogenesis is related to the suppression of small GTPases.

Ⅳ. Discussion

In this study, we demonstrated that ES accelerated the myogenic differentiation. We found that ES increased the thickness and length of myotubes and enhanced the expression of myogenic markers, including MyoD and Myogenin (Fig. 1, 2). In addition, ES activated the fusion of myoblasts during the formation of myotubes (Fig. 3). MyoD and myogenin are members of the MyoD family of transcription factors that have binding interactions with hundreds of muscular gene promoters, playing essential roles in myogenic differentiation [18]. MyoD is capable of converting fibroblasts or other differentiated cell types into the skeletal muscle lineage. Myogenin can induce undergo myogenic differentiation of myoblasts, leading to formation of multinucleated myofibres by fusing of myoblasts [18]. Therefore, our data suggests that ES promotes the myogenic differentiation and myotube formation by increasing MyoD and myogenin.

Previous studies reported the promotive effects of ES on the formation of myotubes by enhancing the expressions of MyoD, M-cadherin, and connexin 43 [18,19]. It was also reported in previous studies using C2C12 cells (mouse myoblast) that ES increased the expression of MHC [19]. In addition, ES pretreatment accelerated the myogenic differentiation of C2C12 cells [17]. However, most studies have used ES to study the effects of exercise on muscle

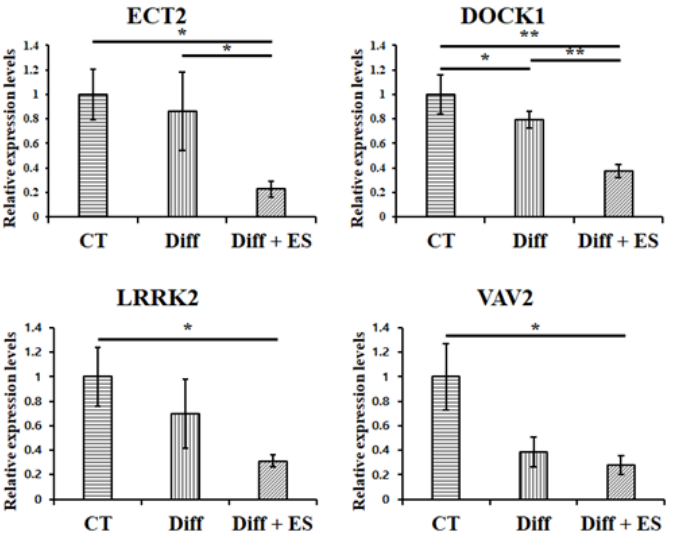

Fig. 4. Electrical stimulation suppresses mRNA expression of small GTPase markers. qPCR analysis of small GTPase, ECT2, DOCK1, LRRK2, and VAV2 in L6s after culture for 7 days in the maintenance medium (CT), differentiation medium (DM), and differentiation medium with ES (ES). Data are presented as the means± standard deviations (S.D.). Statistical analyses were performed using ANOVA (LSD test); **p<.01, *p<.05

cell contraction [15,16]; however, they failed to clearly illustrate the molecular mechanism behind the promotive effects of ES on myogenic differentiation. Small GTPases act as modulators in signal transduction, cell division and growth, vesicle transport, cytoskeletal dynamics, and cell motility [20-23]. Ras GTPases are known to play an important role in human cancer with respect to cellular transformation and proliferation [26-28]. Rho GTPases are primarily involved in cell motility and cytoskeleton dynamics, and control the assembly of stress fibers and focal adhesions [24-29]. In the study using C2C12 myoblasts, RhoA, Rac1, and Cdc42 were found to be important regulators of myogenesis. The RhoA activity increases rapidly and transiently in myoblasts after incubation in the differentiation medium. While RhoA is essential for the initial process of myogenesis, its function must be suppressed prior to myoblast fusion [30]. Active RhoA reduces the stability and disrupts the location of the cell adhesion molecule, M-cadherin, which is essential for muscle fiber fusion [31]. Consistent with the previous results, our results showed that ES promoted the myoblast fusion and down-regulated the expression levels of the small GTPases, such as ECT2, DOCK1, LRRK2, and VAV2 (Fig. 4). These results indicate that ES promotes myoblast fusion by inactivating the small GTPases, ultimately leading to the promotion of myogenic differentiation. In addition, it was known that DOCK1 promotes cell division in various cell types [32]. Thus, it was speculated that ES stimulates myoblast fusion by suppressing cell divisions, inducing myogenic differentiation. However, further study is required to clarify the molecular mechanism underlying the inductive effects of electrical stimulation on myogenic differentiation.

Ⅴ. Conclusion

We demonstrated that electrical stimulation promotes myogenic differentiation. We suggest that ES induces myogenic differentiation by suppressing small GTPase signaling. ES-based techniques can contribute to the

development of a novel therapy for muscle regeneration with high safety, high efficiency, and low cost.

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