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Effect of direct current electrical stimulation on osteogenic differentiation and calcium influx

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Abstract−Electrical stimulation is effective for bone healing. Several studies have shown that electrical stimulation affects cell migration and proliferation. This study examined whether electrical stimulation enhances the osteogenic differentiation of mouse clonal mesenchymal stem cells (cMSCs). Mouse cMSCs were exposed to electrical stimulation at three different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm) for one hour per day. The applied electrical stimulation did not affect the cell viability. The alkaline phosphatase (ALP) activity was increased significantly at day 14 after electrical stimulation with 0.5 V/cm for one hour per day. The genes related to osteogenic differentiation were examined at days three, seven, and 14 after exposure to the three electrical stimuli. Osteocalcin (OC) expression was increased dramatically under all three-voltage conditions. These increases were closely related to the electrically stimulated increases in the intracellular calcium concentrations, assessed by Fluo-4 AM on day 14 after exposure to 0.05 V/cm for one hour per day. Furthermore, the expression of calcineurin/NFAT pathway-related genes was upregulated significantly after electrical stimulation. Thus, electrical stimulation enhances the osteogenic differentiation of mouse cMSCs by increasing the intracellular calcium concentration.

Keywords: Electrical Stimulation, Osteogenic Differentiation, Calcium Flux, Mesenchymal Stem Cells

INTRODUCTION

The need for effective methods to treat bone-related diseases has increased recently, and related studies have been actively pursued. More than half a million patients are treated annually for fractures in the United States. In addition, more than 100,000 new patients suffer from union fractures every year [1]. The cost of treatment amounts to more than $2.5 billion. With the increasing population and life expectancy, this figure is expected to more than double in 2022 [2]. In severe bone-related diseases, such as non-mixed fractures, where parts of the bone are completely lost or severely broken, the treatment period and the cost of treatment are high, highlighting the need for an urgent solution. Despite the existing therapy with autograft or artificial bones, it has a disadvantage of experiencing further damage and recovery [3].

Treatment using MSCs has been suggested to compensate for the shortcomings of artificial bone grafts. MSCs have been used in various tissues for self-renewal and differentiation into various tissues. On the other hand, this may result in MSCs differentiating into unwanted tissues. Hence, it is important to induce differentiation into desired tissues. Previous studies used a method of recombining bone-forming protein genes (such as bmp-2 or bmp-7) as a method for inducing osteogenic differentiation of MSCs [4,5]. On the other hand, other treatment measures are required to replace these proteins owing to the expense and risk of side effects from the dosage used.

Previous researchers studied ways to induce osteogenic differentiation using mechanical simulation as an alternative. Indeed, previous studies have confirmed that mechanical simulation, such as mechanical load, vibration, and ultrasound, affect osteogenic differentiation in mesenchymal stem cells on various scaffolds [6-8]. Electrical stimulation was also studied as a type of mechanical stimulation. In particular, electrical stimulation has already been applied to engineering cardiac or nerve tissues [9,10]. Since the related study reported that electricity has a positive effect on treating bone fractures, efforts have been made to apply electrical stimulation to a range of bone-related diseases [11].

There is a basic charge difference of approximately −15 mV around the cell membrane inside the body. The occurrence of wounds or other stimuli increases the charge difference to approximately 0.2 V due to the movement of various ions [12]. Electrical stimulation controls the activity of cells by artificially producing these charge differences and controlling multiple signals inside the body. Recent studies have shown that biphasic electrical stimulation is effective on the proliferation of mesenchymal stem cells [13]. On the other hand, the mechanism through which electrical stimulation regulates the osteogenic differentiation of cells has not been clarified. This study examined the changes in the osteogenic differentiation and calcium influx due to electrical stimulation.

We designed a system to deliver a direct current (DC) electrical stimulation to cultured mouse MSCs with the 3D printed lid for six-well plates. Using this system, we examined the effects of electrical stimulation on the differentiation of MSCs, which is mediated by changes in intracellular calcium concentration and calcium sig-

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naling mechanism. Optimizing the condition of stimulation for osteogenesis differentiation is the approach to understanding the changes in calcium-mediated differentiation for potential application in bone tissue engineering.

MATERIALS AND METHODS

1. Cell Isolation and Culture

Mouse cMSCs were obtained from SCM Lifescience (Incheon, Korea) [14,15]. Frozen vials of the cells were thawed, cultured, and expanded to reach the desired number, based on the cell provider's instructions. The cMSCs were cultivated in six-well plates at a density of 5,000 cells/cm^2, in the cell growth medium comprising low glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 1% penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). After reaching 80% confluency, the cell growth medium was supplemented with ascorbate (50 μM/ml), dexamethasone (100 nM/L), and β-glycerophosphate (10 mM) and refreshed every two days with this medium. All abbreviation of 'cMSCs' indicated the mouse clonal mesenchymal stem cells obtained from SCM Lifescience, which were used in further all experiments in this study.

2. Electrical Stimulation of cMSCs

The electrical stimulation system consists of a function generator (Key Sight, USA) and fixed platinum electrodes on a plastic lid. The plastic lid was made using a 3D printer (3DP-110F, Cubicon, Korea). The electrodes were placed at a distance of 20 mm and connected to a function generator. Device design of the chamber and arrangement of electrodes (anodes and cathodes) were designed to fit in standard 6-well plate, which was adapted and modified from the device with L-shaped electrode in parallel [16-18]. The cells receiving electrical stimulation were exposed to three different constant voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm) for one hour per day.

3. Cell Viability

The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was applied to determine the effects of electrical stimulation on the cell viability. After electrical stimulation by three different voltages (1 V/cm, 0.5 V/cm and 0.05 V/cm; one hour per day) on days one, seven and 14, MSCs cultivated in six-well plates were incubated with 30 μL of CCK-8 solution at 37 °C for one hour, after which 100 μL of the culture medium was transferred to a 96-well plate. The absorbance was measured at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

4. ALP Activity

cMSCs at a density of 5,000 cells/cm^2 in six-well plates were exposed to three different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm; 1 hr per day). After 14 days of electrical stimulation, the cells were lysed using RIPA buffer supplemented with protease inhibitor. The protein content of the cells was quantified using a BCA assay kit (Thermo Scientific, Waltham, MA, USA). Equal amounts of total protein were placed in 96-well plates, and 100 μl of RIPA buffer was added to each well. p-Nitrophenyl phosphate (pNPP)

Table 1. Primer sequences of osteogenic differentiation and calcium signaling pathway-related genes used in performing RT-qPCR

Target gene	Size (bp[a])	Sequences	Tm[b]
Alkaline phosphatase	163	F: AACAGACCCTCCCCACGAGT	60.5
		R: GTGCCGATGGCCAGTACTAA	57.1
Runx2	174	F: ATTCGTCAACCATGGCCCAG	58.0
		R: AACCCAGTTATGACTGCCCC	57.3
Collagen I_a	139	F: GGCTCTAGAGGTGAACGTGG	57.3
		R: CACCAGGGGCACCATTAACT	57.5
Osteocalcin	81	F: CAGAACAGACAAGTCCCACA	54.6
		R: TCAGCAGAGTGAGCAGAAAGAT	56.0
Osteopontin	167	F: TCCCTCGATGTCATCCCTGT	57.7
		R: ATCACATCCGACTGATCGGC	57.1
Calmodulin	166	F: TGTCCGTGGTGCCGTTACTC	59.7
		R: CATGGTGCGAGCGAAGGAA	58.1
CamkII	167	F: AAACAGCAACCCTTTTGTCCT	55.3
		R: GAGAGACTTCCTCCTTCCACG	57.6
Calcineurin	94	F: GTAGGCACCTCACAGAGTATTT	54.2
		R: CAGTCGAAGGCATCCATACA	54.5
NF-AT	110	F: CCGTCCAAGTCAGTTTCTATGT	54.6
		R: GTCCGTGGGTTCTGTCTTTAT	54.5
β-Actin	238	F: TGTACCCAGGCATTGCTGAC	57.7
		R: AACGCAGCTCAGTAACAGTCC	57.3

[a]Base pair
[b]Melting temperature (°C)

was used to quantify the alkaline activity (Sigma Aldrich, USA). Briefly, 50 μl pNPP was added to the wells containing the proteins. After 30 minutes of incubation, the reaction was quenched by adding 50 μl 3 M NaOH, and analyzed at 405 nm.

5. Gene Expression Analysis

Real-time quantitative PCR was applied to analyze the effects of electrical stimulation on gene expressions of the osteogenic differentiation-related proteins and Ca^{2+}-calcineurin/NFAT signaling proteins. After three, seven, and 14 days of exposure to electrical stimulation by three different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm; one hour per day), the total RNA of MSCs was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. The concentration of extracted RNA was determined using a microspectrophotometer (DS-11, DeNovix, Wilmington, DE, USA). Each RNA sample was synthesized to cDNA using a PrimeScript RT reagent kit (Takara, Shiga, Japan). RT-qPCR was performed using a CFX96 detection system (Bio-Rad, Hercules, CA, USA) with PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Beverly, MA, USA). The target genes were RUNX2, alkaline phosphatase (ALP), collagen Ia (COL Ia), osteocalcin (OC), osteopontin (OPN), calcineurin (CaN), calmodulin-dependent protein kinase II (CaMK II), and nuclear factor of activated T-cells (NFAT). β-Actin was used as the housekeeping gene. Table 1 lists the primers for the target genes. The relative mRNA expression levels were calculated with respect to β-actin and static control group using the $2^{(-\Delta\Delta CT)}$ method.

6. Calcium Fluorescence Assay

The intracellular Ca^{2+} levels were detected after 14 days of electrical stimulation with 0.05 V/cm (1 hr/day) using the Fluo-4 AM calcium assay kit (Life Technologies, CA, USA). The calcium concentration was measured according to the manufacturer's instructions. Briefly, 2 ml Fluo-4 AM dye loading solution (10 μl in 10 ml DPBS) was added to each well. The plates were incubated at 37 °C for 30 minutes, followed by 30 minutes incubation at room temperature. The stained samples were then rinsed with DPBS and mounted with a mounting solution (Vectashield H-1200, Vector Laboratories, Burlingame, CA, USA) containing DAPI for counterstaining. The fluorescence images were obtained using an inverted microscope (CKX 53, Olympus, Tokyo, Japan) with a charge-coupled device camera (DP80, Olympus). The images were processed using the cell Sens software Olympus), setting the excitation and emission wavelengths to 494 nm and 530 nm, respectively.

7. von Kossa Assay

von Kossa staining was used for comparing the mineralization of cMSCs. Briefly, cMSCs were plated in six-well plates and cul-

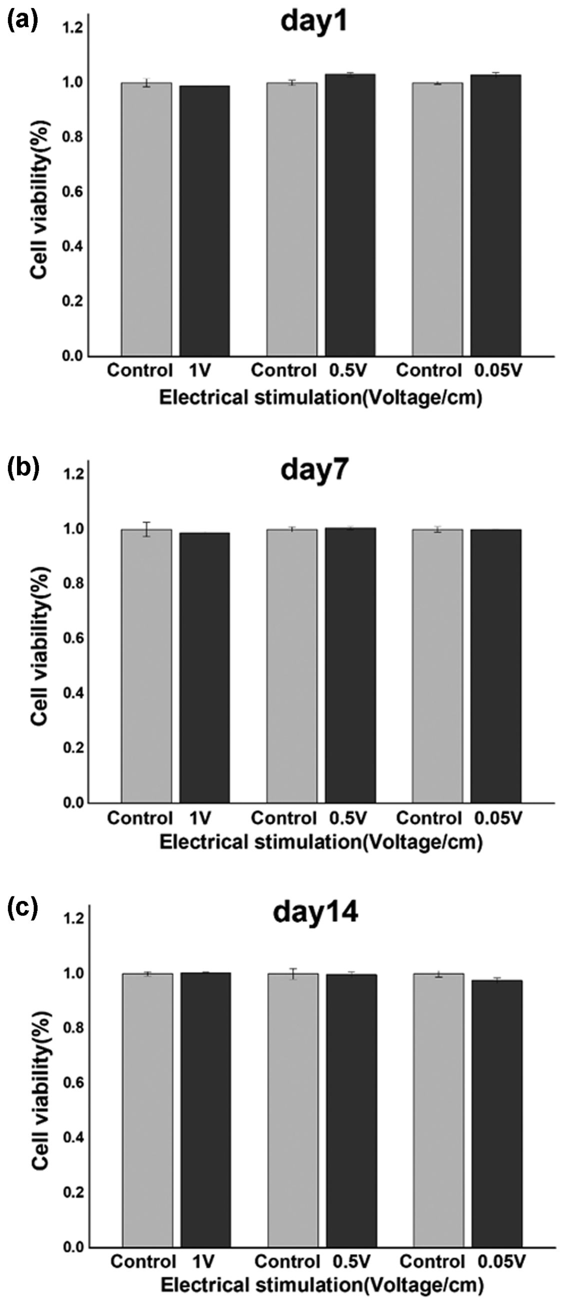

Fig. 1. Cell viability of MSCs after exposure to electrical stimulation. Day 1 (a), day 7 (b), and day 14 (c). Relative cell viability ratio was calculated by normalizing the absorbance of the electrical stimulation group to that of the control group (n=3).

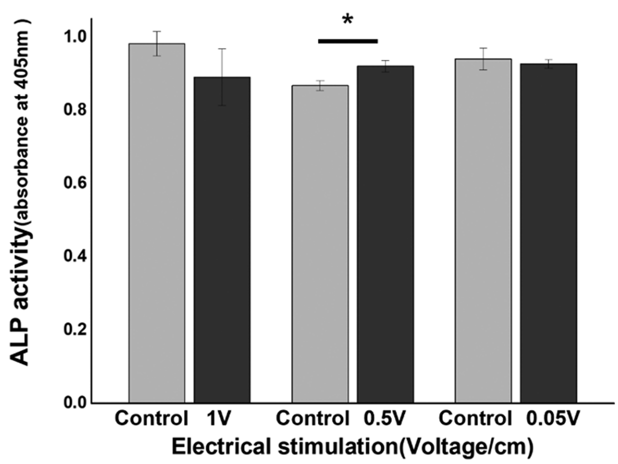

Fig. 2. Alkaline phosphatase (ALP) activity of cMSCs. MSCs were exposed to three different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm) of electrical stimulation for 14 days. There was a significant increase in ALP activity at voltage 0.5 V/cm. * $p < 0.05$.

tured in an osteogenic differentiation medium with and without electrical stimulation (0.05 V/cm, one hour/day) for 14 days. The von Kossa stain kit (Abcam, UK) was used to measure calcium deposition. The cultured cells were washed with DPBS and fixed with 4% paraformaldehyde (Sigma Aldrich, München, Germany) for 15 minutes. A 5% silver nitrate solution was added to the fixed cells and incubated under UV light for 1 hr. The cells were then washed with deionized water, and images were captured optical microscopy (CKX53, cellSens Entry 1.9 Software; Olympus, Tokyo, Japan).

8. Statistical Analysis

A cell viability assay, ALP activity assay, and RT-qPCR were re-

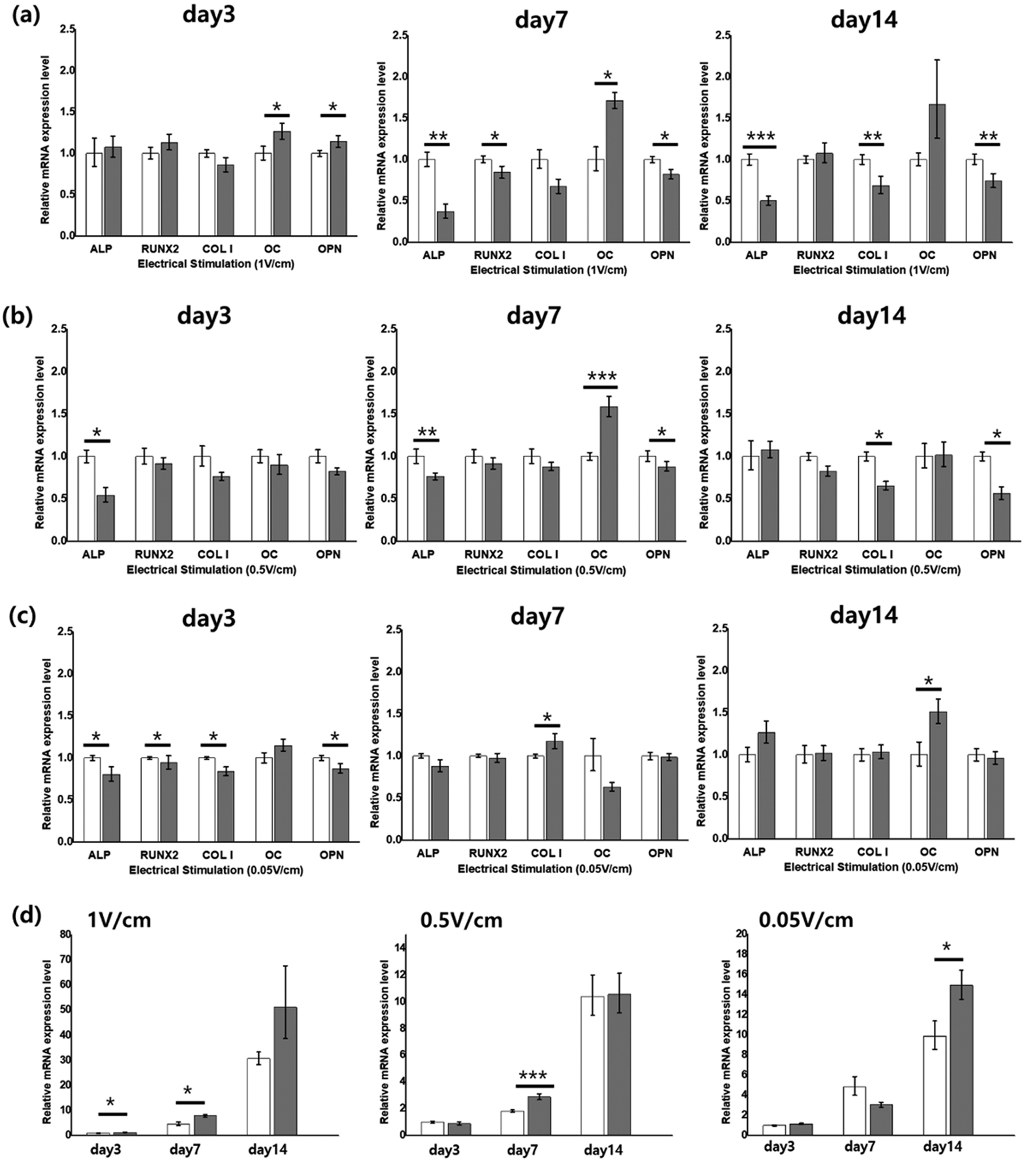

Fig. 3. Gene expression levels of osteogenic differentiation-related genes (RUNX2, ALP, COL Ia, OC and OPN) of MSCs, following exposure to electrical stimulation with 1 V/cm (a), 0.5 V/cm (b), and 0.05 V/cm (c). Osteocalcin expression of MSCs following exposure to electrical stimulation on different days (d). Control group (white) and electrical stimulation group (grey) * p<0.05, ** p<0.01, and *** p<0.001.

peated at least three times independently. The results are displayed as the mean±standard error of the mean values. A Student's t-test (two-tailed, equal variance) was used to identify the significance of differences between the electrical stimulation exposed group and the static control group.

RESULTS

1. Cell Viability

The cell counting kit-8 (CCK-8) assay was used to measure the viability of cMSCs after electrical stimulation at different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm) (Fig. 1). On days one and seven, the applied electrical stimulation exerted no significant changes in cell viability. On day 14, the electrically stimulated group (0.05 V/cm) showed a slight decrease in cell viability, but the difference was not significant (Fig. 1(c)).

2. ALP Activity

The ALP activity was evaluated to examine the effects of electrical stimulation on osteogenic differentiation of cMSCs. The cells were cultured for 14 days with three different electrical stimulations. No significant change was observed in the ALP activity of MSCs after exposure to electrical stimulation at 1 V/cm and 0.05 V/cm. On the other hand, electrical stimulation at 0.5 V/cm showed a significant increase in ALP activity ($p<0.05$) compared to the control group and was determined to be 7% higher than the values obtained in the control group (Fig. 2).

3. Osteogenic Differentiation Gene Expression

Osteogenic differentiation-related genes (RUNX2, alkaline phosphatase (ALP), collagen Ia (COL Ia), osteocalcin (OC), and osteopontin (OPN)) were analyzed by RT-qPCR on days three, seven, and 14 to measure gene expression alterations after exposure to electrical stimuli. Electrical stimulation with 1 V/cm altered OC and OPN expression significantly on day three. On days seven and 14, significant changes were observed in the expressions of ALP, RUX2, OC, and OPN (Fig. 3(a)). Electrical stimulation with 0.5 V/cm significantly altered only ALP expression on day three. On days seven and 14, ALP, COL Ia, OC, and OPN expression was changed significantly. In particular, OC expression was significantly higher than the control after electrical stimulation at 0.5 V/cm (Fig. 3(b)). On day three, electrical stimulation with 0.05 V/cm altered all differentiation markers significantly, except for OC. On day seven, only COL Ia expression was significantly higher than the control. On day 14, only OC expression was significantly higher than the control after exposure to electrical stimulation with 0.05 V/cm (Fig. 3(c)). The overall results indicate that osteocalcin expression is significantly higher than the control on day seven, after exposure to electrical stimulation with 1 V/cm and 0.5 V/cm. In contrast, osteocalcin expression is significantly higher than the control on day 14 after exposure to electrical stimulation with 0.05 V/cm (Fig. 3(d)).

4. von Kossa Assay

A von Kossa assay was applied to measure the changes in the mineralization of cMSCs, after exposure to electrical stimulation with 0.05 V/cm (Fig. 4). Calcium deposition was observed microscopically after 14 days of electrical stimulation with 0.05 V/cm. More areas with calcium deposits were detected in cMSCs after electrical stimulation exposure compared to the control (Fig. 4(b)).

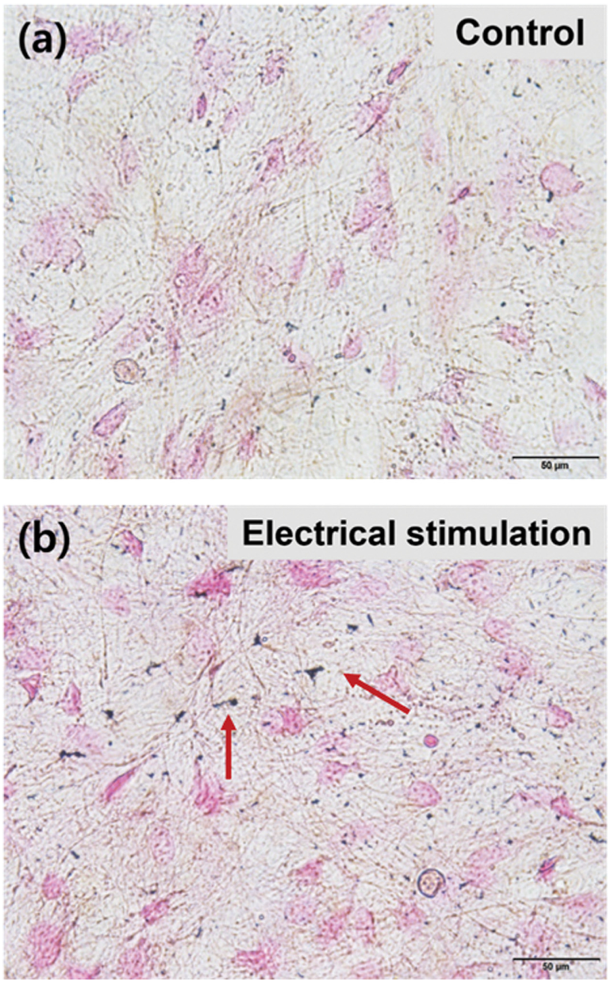

Fig. 4. Mineralization of MSCs on day 14, following exposure of electrical stimulation with 0.05 V/cm. Calcium deposits of the mineralized matrix were observed with von Kossa staining. Control group (a) and electrical stimulation group (b). The calcium deposited area appears black and nuclei are stained red.

5. Calcium Fluorescence Assay

A calcium fluorescence assay was performed to measure the alterations in the concentration of intracellular calcium in MSCs following exposure to electrical stimulation with 0.5 V/cm at day 14 (Fig. 5). Intracellular calcium concentration of MSCs was observed to increase after exposure to electrical stimulation with 0.05 V/cm at day 14 (Fig. 5(b)).

6. Calcium Signaling Pathway-related Gene Expression

The expression of the genes related to the calcium-signaling pathway (Calmodulin, CaMKII, Calcineurin, and NF-AT) was analyzed on days three, seven, and 14. On days three and seven, electrical stimulation with 0.05 V/cm resulted in no significant changes in gene expressions (Fig. 6). On day three, there was increased CaMKII expression, but the difference was not statistically significant. On the other hand, the expression of all calcium signaling-related genes after exposure to electrical stimulation was higher than the control group on day 14. In particular, the expression of calcineurin and NF-AT were increased significantly.

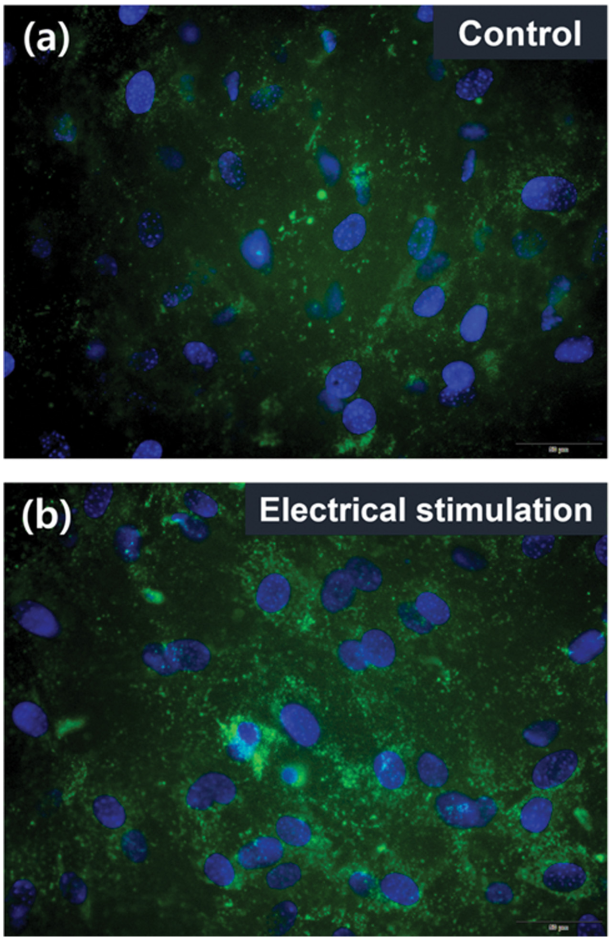

Fig. 5. After electrical stimulation exposure with 0.05 V/cm, intracellular Ca^{2+} concentration was detected using Fluo-4 AM with DAPI for counterstaining in MSCs at day 14. Control group (a) and electrical stimulation group (b).

DISCUSSION

Electrical stimulation is an effective tool for various tissues [19-21]. Recently, the effects of electrical stimulation on MSCs have been reported [22]. Numerous studies suggest that electrical stimulation affects cell migration and induces osteogenic differentiation [23,24]. This study examined the effects of DC electrical stimulation on the viability, ALP activity, osteogenic differentiation marker genes, and calcium flux of cMSCs. These results show the potential of electrical stimulation on osteogenic differentiation and indicate the correlation between the calcium flux and the osteogenesis of MSCs.

A wide range of voltages ranging from 0.05 V/cm to 10 V/cm was evaluated to optimize the stimulation conditions. Not all voltages were feasible in this study because of bubble and heat generation over 1 V/cm in the culture (data not shown). Thus, three different voltages (1 V/cm, 0.5 V/cm, and 0.05 V/cm) were finally included. The data indicate that electrical stimulation in these ranges does not affect the cell viability (Fig. 1). The cellular ALP activity was increased significantly after 14 days of electrical stimulation at 0.5 V/cm without any effects on cell viability, which could reflect

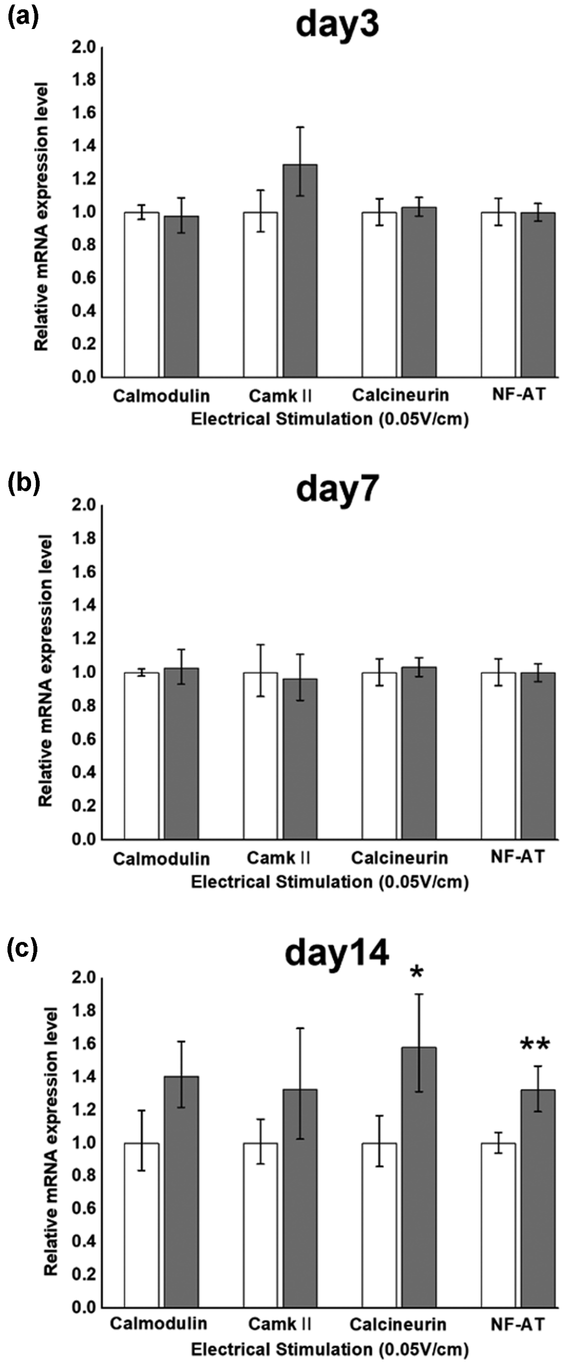

Fig. 6. Gene expression of calcium signaling pathway-related genes (Calmodulin, Camk2, Calcineurin, and NF-AT) of cMSCs, following exposure to electrical stimulation with 0.05 V/cm, at days 3, 7, and 14. The control group (white) and electrical stimulation group (grey) were compared. * $p<0.05$, ** $p<0.01$.

the osteogenic ability of MSCs (Fig. 2). In addition to the assay related to the cellular ALP activity, the gene expression level of the osteogenic differentiation markers, such as RUNX2, ALP, COL Ia, OC, and OPN, were also analyzed to examine the effect of electrical stimulation (Fig. 3). The expression of the osteogenic differentiation early-stage marker RUNX2 remained unchanged at days three, seven, and 14. Moreover, ALP did not change at 0.05 V/cm

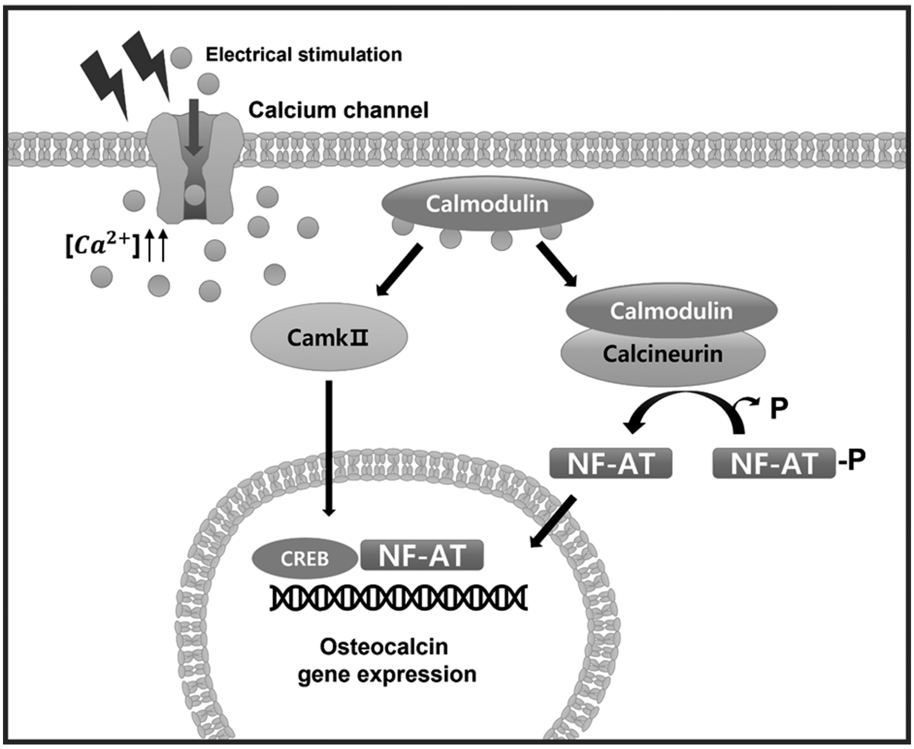

Fig. 7. Calcium signaling pathway. Electrical stimulation alters the intracellular calcium concentration. Increased calcium ion activates the calcium signaling pathway, which induces gene expression of osteocalcin.

but decreased significantly at 1 V/cm. The decreased level of ALP gene expression at 1 V/cm was possibly due to changes in the reactive oxygen species (ROS) generated in the medium by electrical stimulation. According to a previous paper, the transmembrane potential was excessively changed at high voltage [25], resulting in increased ROS in the medium [26]. ROS alters the medium pH, resulting in decreased ALP gene expression [27]. On the other hand, ALP activity (Fig. 2) showed no significant decrease at 1 V/cm, indicating that the decreased ALP gene expression does not affect the protein activity. In contrast, at days seven and 14, the late-stage marker (OC) was increased following exposure to electrical stimulation under all voltage conditions. Non-collagenous protein osteocalcin plays an essential role for regulating bone mineralization and bone turnover by having high affinity with calcium. While a related mechanism still remains unclear, it was reported that osteocalcin could enhance the bone formation and remodeling [28,29]. These results of gene expression analysis appear to be related to altered calcium concentration after electrical stimulation. Therefore, further experiments were conducted with cMSCs exposed to electrical stimulation at 0.05 V/cm for 14 days to determine the correlation between the related calcium flux and late-stage differentiation. The changes in the intracellular calcium concentration of cMSCs were analyzed using Fluo-4 AM, following exposure to electrical stimulation at 0.05 V/cm for 14 days. Calcium fluorescent images suggest that the calcium concentration increases after electrical stimulation compared to before (Fig. 5).

Calcium acts as a signal molecule in several pathways, e.g., calcium-calcineurin/NFAT signaling. Calcium-calcineurin/NFAT signaling affects the process of osteogenic differentiation (Fig. 7).

Calcium activates the calcineurin/NFAT signaling pathway, which increases the level of osteogenic differentiation-related genes [30]. RT-qPCR of the calcineurin/NFAT signaling pathway-related markers (Calcineurin, CaMK II, NF-AT) (Fig. 6) revealed upregulated levels compared to the control group, after 14 days of electrical stimulation at 0.05 V/cm. Hence, electrical stimulation affects the expression level of osteogenic differentiation osteocalcin by affecting the intracellular calcium concentration. In addition, electrical stimulation induces the mineralization of cMSCs, by demonstrating the areas with calcium deposition (Fig. 4). No significant difference in the early stages of electrical stimulation and osteogenic differentiation was observed, but a large change was detected in the late stage, which could be attributed to several reasons. The increase in calcium ions by electrical stimulation inhibits the early osteogenic differentiation process and thereby affects ossification-related genes [27]. In addition, because the microenvironment is different for each cell in the *in vitro* experiment, the osteogenic differentiation in the early-stage and calcium signaling-related genes did not change when electrical stimulation was applied, but in the *in vivo* experiment, the genes were upregulated after electrical stimulation [31]. The aforementioned limitations of *in vitro* direct electrical stimulation were also well discussed through the computational current density magnitude distribution in the reference [32]. Therefore, further studies will be needed to optimize the stimulation system by minimizing the effects of variations in microenvironment or by-products, such as ROS from electrically stimulated media, and examine the *in vivo* alterations in clinical application. Although further studies will be needed to overcome this, these results highlight the therapeutic potential of electrical stimulation in tissue engineering for osteogenic differentiation via a calcium-mediated mechanism.

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