Monophasic pulsed 200 μA current promotes galvanotaxis with polarisation of actin filament and integrin α2β1 in human dermal fibroblasts

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(Degree)
博士（保健学）

(Date of Degree)
2016-03-25

(Date of Publication)
2017-03-01

(Resource Type)
doctoral thesis

(Report Number)
甲第6615号

(URL)
https://hdl.handle.net/20.500.14094/D1006615

Mikiko Uemura

博　士　論　文

Monophasic pulsed 200 μA current promotes galvanotaxis with polarisation of actin filament and integrin α2β1 in human dermal fibroblasts

（200 μA の単相性パルス電流はヒト皮膚由来線維芽細胞の integrin α2β1 およびアクチン線維を分極させて遊走を促進する）

平成 28 年 1 月 18 日

神戸大学大学院保健学研究科保健学専攻

植村　弥希子

Abstruct

Objective: The monophasic pulsed micro-current (MPMC) is used to promote wound healing, and galvanotaxis regulation has been reported as one of the active mechanisms in promotion of tissue repair with MPMC. However, the optimum MPMC parameters and intracellular changes caused by MPMC have not been elucidated in human dermal fibroblasts (HDFs). The purpose of this study was to investigate the optimum intensity for promoting galvanotaxis and the effects of electrical stimulation on integrin α2β1 and actin filaments in HDFs.
Methods: HDFs were treated with MPMC of 0, 100, 200, or 300 μA for 8 h, and cell migration and cell viability were measured 24 h after starting MPMC stimulation. Polarisation of integrin α2β1 and lamellipodia formation was detected by immunofluorescent staining 10 min after starting MPMC stimulation.
Results: The migration towards the cathode was significantly higher in the cells treated with the 200 μA MPMC than in the controls ($p<0.01$) without any change in cell viability; treatment with 300 μA MPMC did not alter the migration ratio. The electro-stimulus of 200 μA also promoted integrin α2β1 polarisation and lamellipodia formation at the cathode edge ($p<0.05$).
Conclusion: The results show that 200 μA is an effective MPMC intensity to promote migration towards the cathode, and this intensity could regulate polarisation of migration-related intracellular factors in HDFs.

Title

Monophasic pulsed 200 μA current promotes galvanotaxis with polarisation of actin filament and integrin α2β1 in human dermal fibroblasts

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Introduction

Electrical stimulation is recommended for treating pressure ulcers (PU) in Prevention and Treatment of Pressure Ulcers: Clinical Practice Guideline edited by the National Pressure Ulcer Advisory Panel, European Pressure Ulcer Advisory Panel, and Pan Pacific Pressure Injury Alliance in 2014[1] and monophasic pulsed micro-current (MPMC) stimulation is one of effective electrical therapy. Several clinical studies[2-4] have reported that MPMC stimulation shortened the healing period for PU; however, the parameters of MPMC, including intensity and polarity, are not identical in each clinical study.

Migration of fibroblasts towards the wound site is important to promote the formation of granulation tissue[5], and galvanotaxis is a crucial factor to promote migration. Our previous study[6] showed that migration towards cathode was greater in human dermal fibroblasts (HDFs) treated with 100 μA MPMC than in controls treated with 0 μA MPMC. Furthermore, PU healing was promoted by MPMC of 50-100 μA with the cathode contacting the wound site in our clinical studies[7-8]. These studies suggested that cathodal

micro-current intensity promotes galvanotaxis of fibroblasts towards the cathode, as well as wound healing. However, the intracellular change induced by MPMC stimulation, and the effects of current intensities greater than 100 μA are not clear.

Integrin is a cell surface adhesion protein that activates outside-in signalling, leading to regulation of several cellular functions including cytoskeletal dynamics[9]. In fibroblasts, integrin α2β1 binds to collagen and mediates migration and fibrosis[10]. Integrin-triggered signalling induces actin polymerization such as lamellipodia at the leading edge[11]. When cells migrate, lamellipodia formation occurs on the leading edge and initiates migration[12]. Therefore, regulation of lamellipodia formation can affect cell migration. However, no study has investigated the effect of MPMC on lamellipodia formation in HDFs.

We hypothesized that HDFs migrate towards the cathode by MPMC stimulation and that optimum intensity for migration could alter migration-related intracellular factors such as integrin α2β1 and lamellipodia. For effective and safe treatments, it is important to elucidate the influence of MPMC on cell dynamics and intracellular alteration. Therefore, we examined the optimum intensity of MPMC for cell migration and the effects of MPMC on integrin α2β1 polarisation and lamellipodia formation in HDFs.

Methods

Cell culture

HDFs (CC-2511; Clonetics, San Diego, CA, USA) were grown in Dulbecco's Modified Eagle's Medium (Wako, Osaka, Japan) supplemented with 10% foetal bovine serum (Nichirei, Tokyo, Japan) in 100 mm tissue culture dishes (Iwaki, Tokyo, Japan) in a CO_2 incubator at 37℃. Fibroblasts that had undergone 3-7 passages were used for the experiments.

Cell migration assay

Cover glasses (Cytograph L60S300; Dai Nippon Printing, Tokyo, Japan) were attached to the centre of 100 mm tissue culture dishes and covered with a film that had a 14 mm hole in the centre. The cover glasses contained 60 μm-wide grooves for cell attachment that were divided by 300 μm-wide non-cell-adherent areas, which was the best width for observing fibroblast migration in the preliminary study. For the experiments, fibroblasts (1.63×10^4 cells) were seeded on the cover glass in a circular pattern through the hole at the centre of the cover film and cultured for 24 hours (Figure 1a) according to the

manufacturer's instruction. To identify the baseline for measuring the migration distance, the baseline was marked with a point on the bottom of the dishes. After incubation for 24 hour, the cover film was removed, the dish was filled with culture medium, and the electrical stimulation experiment was conducted with a dedicated electrical stimulation device (Figure 1b). The electrical stimulation procedure was performed as in our previous study[6]. Platinum electrodes (20 × 5 mm plates) were used to prevent metal ion toxicity. The MPMC (frequency, 0.3 Hz; pulse duration, 250 msec) stimulation of the fibroblasts was conducted in a CO_2 incubator at 37°C for 8 hours with current intensities of 0 (control), 100, 200, and 300 μA. Since reverse current could be generated after monophasic pulsed current stimulation[6], we connected the anode and cathode with electrical ground cable for 1 minute after the MPMC stimulation to prevent this potential side effect in the present study. To analyse cell migration induced by MPMC, the cells were observed with a microscope under 50× magnification (Axiovert 25; Carl Zeiss, Oberkochen, Germany). Images were taken before MPMC stimulation and 24 h afterwards with a digital camera (Camedia c-5050 zoom; Olympus, Tokyo, Japan), and the pictures were synchronized with computer software (e-Tiling). A baseline perpendicular to the grooves was drawn from the initial point placed on the back of the dishes. For each group, we analysed 10 grooves in the centre of the cell attachment area and measured the distances of cell attachment area from the baseline towards the anode or cathode. To assess the migration, we calculated the distances between the cell attachment area before and after the MPMC stimulation to obtain the migration distances. The results were expressed as a migration rate, a ratio of the migration distance towards the cathode versus towards the anode.

Trypan blue-exclusion test

We used a trypan blue-exclusion assay to assess the cell number and viability following the MPMC. The 25 mm cover glasses (Matsunami, Osaka, Japan) were placed in each well of a 6-well plate (Iwaki), and fibroblasts (28×10^4 cells/well) were seeded into the well and cultured for 24 hours, after which we transferred the cover glass to the centre of a 100 mm culture dish. The fibroblasts were electro-stimulated for 8 hours in the incubator and the assay was conducted at 8 hour after the start of electrical stimulation. The number of living and dead cells was counted using a haemocytometer under a light microscope.

Immunofluorescence staining

Fibroblasts (5×10^4 cells/well) were seeded into the 25 mm cover glasses placed in the well of a 6-well plate and cultured for 2 hours. Next, the cells received MPMC stimulation for 10 minutes in a CO_2 incubator with an intensity of 0, 100, 200, or 300 μA. Fibroblasts were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X, and then incubated with mouse anti-integrin α2β1 antibody, clone BHA2.1 (1:50, Merck Millipore, Darmstadt, Germany) overnight at 4°C. Cells were washed with PBS and incubated with Alexa Fluor 594 goat anti-mouse IgG (H+L) secondary antibody (1:500, Life Technologies, Carlsbad, CA, USA) for 1 hour at room temperature. Following this staining, phalloidin-Alexa Fluor 488 (1:40, Life Technologies, Carlsbad, CA, USA) was applied for 20 minutes and DAPI (1:1000, Dojindo, Kumamoto, Japan) for 3 minutes at room temperature. The coverslip was mounted with mounting medium (Fluorescent Mounting Medium, DAKO, Carpinteria, CA, USA). The stained cells were observed with a fluorescence microscope (Axio Vert. A1, Carl Zeiss, Oberkochen, Germany).

Integrin α2β1 polarisation assay

The central 10 fields were selected in each sample. To quantify integrin α2β1 polarisation, we divided each cell membrane in half, based on the side adjacent to the cathode or the anode, and demarcated the area using an image tool (Image J). In each cell, the average fluorescence intensities for integrin α2β1 of cathode-adjacent (CA) and anode-adjacent (AA) cell membrane were corrected for whole average intensity. Asymmetry index was calculated and used to analyse the polarisation according to Finkelstein's study[13].

Asymmetry index = (average of AA – average of CA) / average of whole cell

We calculated asymmetry index in each cell and the average of all the cells.

Lamellipodia formation

We observed lamellipodia formation in the same fields that were analysed for asymmetry index. We classified F-actin-rich membrane extensions as lamellipodia according to Steffen's study[14]. The ratio of lamellipodia formation at cathode facing to that at anode facing was calculated in each sample by counting the number of lamellipodia in each edge under the fluorescence microscope (100× magnification).

Statistical analysis

The differences in the migration ratios, cell numbers, cell viabilities, asymmetry index, and lamellipodia formation ratio between the control group and the electro-stimulus groups were analysed with a Tukey-Kramer multiple comparisons test. Differences with $p<0.05$ were considered to be significant.

Results

Monophasic pulsed micro-current promotes galvanotaxis in human dermal fibroblasts

In MPMC groups, fibroblasts migrated towards the cathode (Figure 2). The migration ratio for the 0, 100, 200, and 300 μA groups was 0.93 ± 0.13, 1.27 ± 0.25, 2.65 ± 0.37, and 1.19 ± 1.70, respectively (Figure 3). At 200 μA, fibroblast migration towards the cathode was significant ($p<0.01$), but at 300 μA, the migration ratio decreased to less than the ratio at 100 μA. To investigate whether the high migration ratio in the 200 μA group and the decrease in the 300 μA group were caused by cell proliferation or cell toxicity by MPMC, we analysed cell number and viability in the 200 and 300 μA groups using the trypan blue assay. There were no appreciable differences in the cell numbers or viability between the 0 μA group and MPMC groups (Figure 4). Therefore, the increase in the migration ratio was caused by the MPMC promoting the migration of the fibroblasts.

Integrin α2β1 polarisation

A negative asymmetry index indicates that integrin α2β1 polarises to the cathode. In the 0 μA group, integrin α2β1 was observed over the entire cell surface, while with MPMC groups, integrin α2β1 polarised to the edge of the cell as shown in Figure 5a, d, g, j. Figure 6a shows that integrin α2β1 polarisation to the cathode is significantly higher in the 200 μA group than in the 0 μA group as determined by analysis of asymmetry index ($p<0.05$). Similar to the result for migration, when stimulated with 300 μA, integrin α2β1 did not polarise to the cathode.

Lamellipodia formation in electrical stimulation

We next investigated whether MPMC influences actin organization by staining for F-actin. Lamellipodia, the projections on the leading edge of the cell, propel cell migration. Figure 5b, k shows that lamellipodia were formed at both the cathode and anode edge in the 0 and 300 μA groups, whereas in the 200 μA group, they were observed at the cathode

edge (Figure 5h). The ratio of lamellipodia formation at the cathode edge versus the anode edge was significantly higher in the 200 μA group than in the 0 and 300 μA groups (Figure 6b, $p<0.05$). Lamellipodia polarisation stimulated by 200 μA indicates that MPMC at this intensity can directly affect the assembly of actin filaments.

Discussion

We found that MPMC of 200 μA promoted galvanotaxis of HDFs towards the cathode. Moreover, it appeared that the integrin α2β1 polarisation and lamellipodia formation observed at the cathode edge are related to μA intensity up to 200 μA. These findings suggest that the MPMC of optimum intensity and polarity are important to promote migration of fibroblasts, and the effective intensity in migration could regulate the polarisation of migration-related intracellular factors.

MPMC of 200 μA strongly increased migration ratio towards the cathode; however, stimulation of 300 μA demonstrated a lower migration ratio than 200 μA, and a ratio similar to the 100 μA group. Although some *in vitro* studies[15-18] also showed that fibroblasts migrated towards the cathode by electrical stimulation, these studies did not assess the dependency on intensity in the migration. The present study revealed an optimum micro-current intensity of 200 μA to promote galvanotaxis of HDFs with the single peak change of migration. This single peak observed in HDFs is in agreement with the study using neutrophils showing that migration distance was inhibited at intensities greater than the optimum intensity of 60 μA[19]. These results suggest that each cell type has an optimum intensity for galvanotaxis.

With an MPMC of 200 μA, polarisation of integrin α2β1 and lamellipodia were observed at the cathode edge. Our results for integrin polarisation by MPMC agree with the results in ligament fibroblasts assessed by difference in volts[15]. Meanwhile, at amplitudes of 100 and 300 μA, polarisation of integrin α2β1 and lamellipodia towards the cathode was not observed. The present study is the first demonstration of these single peak changes in migration-related intracellular factors in HDFs by MPMC stimulation. Moreover, the migration assay and immunofluorescence analysis found consistency in the optimum MPMC intensity between promotion of migration and polarisation of migration-related intracellular factors. Actin polymerization at the leading edge is necessary for cell migration, and Li et al[20] demonstrated that an electric field induced direct migration of bovine vascular endothelial cells to the cathode and concentration of F-actin at the cathode. Although we could not analyse the lamellipodia formation of the

HDFs in the migration assay, the action of MPMC on lamellipodia polarisation may promote cell migration. Focal adhesion kinase (FAK) is one of the scaffold proteins of integrin. Small electric fields were shown to activate FAK[21], and integrin-activated FAK leads to lamellipodia formation and cell migration[22]. These results suggest that MPMC polarised integrin α2β1 at the cathode edge; then, outside-in signalling occurred, leading to actin polymerization. Therefore, integrin α2β1 may play a role as a mechanosensor of MPMC. However, the relationship between migration and both integrin α2β1 polarisation and lamellipodia formation are still incompletely understood in the present study. Further studies are needed to investigate other factors of outside-in signalling, including FAK.

This study revealed that MPMC of 200 μA influenced *in vitro* galvanotaxis of fibroblasts towards the cathode in accordance with distribution of integrin α2β1 and actin filaments. Indeed, *in vivo* research has shown that measurable micro-current generated following mammalian skin injury accompanies tissue healing[23]. Therefore, in clinical trials, it is important to determine the current intensity, polarity, and state of target tissues for healing PU and other chronic wounds. Based on the results of the present study, future investigations should be conducted to determine the effective parameters on the frequency and treatment time, and the influence on integrin-triggered related signalling.

Acknowledgements

This study was partially supported by JSPS KAKENHI Grant Number 22500458 and by Japanese Society of Pressure Ulcers Research grant 2015.

References

1. National Pressure Ulcer Advisory Panel (NPUAP), European Pressure Ulcer Advisory Panel (EPUAP) and Pan Pacific Pressure Injury Alliance (PPPIA). Prevention and Treatment of Pressure Ulcers: Clinical Practice Guideline. Washington, DC: National Pressure Ulcer Advisory Panel;2014:47.
2. Kawasaki L, Mushahwar VK, Ho C, Dukelow SP, Chan LL, Chan KM. The mechanisms and evidence of efficacy of electrical stimulation for healing of pressure ulcer: a systematic review. Wound Repair Regen. 2014.22:161-73
3. Gardner SE, Frantz RA, Schmidt FL. Effect of electrical stimulation on chronic wound healing: a meta-analysis. Wound Repair Regen. 1999.7:496–3.

4. Lala D, Spaulding SJ, Burke SM, Houghton PE. Electrical stimulation therapy for the treatment of pressure ulcers in individuals with spinal cord injury: a systematic review and meta-analysis. Int Wound J. In Press.
5. Schreier T, Degen E, Baschong W. Fibroblast migration and proliferation during in vitro wound healing. A quantitative comparison between various growth factors and a low molecular weight blood dialysate used in the clinic to normalize impaired wound healing. Res Exp Med (Berl). 1993.193:193-205.
6. Sugimoto M, Maeshige N, Honda H, Yoshikawa Y, Uemura M, Yamamoto M, Terashi H. Optimum microcurrent stimulation intensity for galvanotaxis in human fibroblasts. J Wound Care. 2012.21:5–11.
7. Honda H. Sugimoto M. Maeshige N. Terashi H. Efficacy of microcurrent stimulation for pressure ulcer healing—a single case design. Jpn J PU. 2014;14:63-7. [In Japanese]
8. Yoshikawa Y. Sugimoto M. Maeshige N. Terashi H. Clinical application of direct micro-current stimulation to sacral pressure ulcer. Jpn J PU. 2012.13:582-6. [In Japanese]
9. Plotnikov SV, Waterman CM. Guiding cell migration by tugging. Curr Opin Cell Biol. 2013.25:619-26.
10. Eckes B, Zweers MC, Zhang ZG, Hallinger R, Mauch C, Aumailley M, Krieg T. Mechanical tension and integrin alpha 2 beta 1 regulate fibroblast functions. J Investig Dermatol Symp Proc. 2006.11:66-72.
11. Brown MJ, Loew LM. Electric field-directed fibroblast locomotion involves cell surface molecular reorganization and is calcium independent. J Cell Biol. 1994127:117-28.
12. Lauffenburger DA, Horwitz AF. Cell Migration: A Physically Integrated Molecular Process. Cell. 1996.84:359-69.
13. Finkelstein EI, Chao PH, Hung CT, Bulinski JC. Electric field-induced polarization of charged cell surface proteins does not determine the direction of galvanotaxis. Cell Motil Cytoskeleton. 2007.64:833-46.
14. Steffen A, Ladwein M, Dimchev GA, Hein A, Schwenkmezger L, Arens S, Ladwein KI, Margit Holleboom J, Schur F, Victor Small J, Schwarz J, Gerhard R, Faix J, Stradal TE, Brakebusch C, Rottner K. Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. J Cell Sci. 2013.126:4572-88.
15. Tsai CH. Lin BJ. Chao PH. α2β1 integrin and RhoA mediates electric field-induced ligament fibroblast migration directionality. J Orthop Res. 2013.31:322-7.

16. Kim MS, Lee MH, Kwon BJ, Koo MA, Seon GM, Park JC. Golgi polarization plays a role in the directional migration of neonatal dermal fibroblasts induced by the direct current electric fields. Biochem Biophys Res Commun. 2015.460:255-60.
17. Choi H, Cho JS, Park IH, Yoon HG, Lee HM. Effects of microelectrical current on migration of nasal fibroblasts. Am J Rhinol Allergy. 2011.25:157-62.
18. Chao PH, Lu HH, Hung CT, Nicoll SB, Bulinski JC. Effects of applied DC electric field on ligament fibroblast migration and wound healing. Connect Tissue Res. 2007.48:188-97.
19. Fukushima K, Senda N, Inui H, Miura H, Tamai Y, Murakami Y. Studies of galvanotaxis of leukocytes. I. Galvanotaxis of human neutrophilic leukocytes and method of its measurement. Med J Osaka Univ. 1953;4:195–8.
20. Li X, Kolega J. Effects of direct current electric fields on cell migration and actin filament distribution in bovine vascular endothelial cells. J Vasc Res. 2002.39:391-4.
21. Zhang J, Ren R, Luo X, Fan P, Liu X, Liang S, Ma L, Yu P, Bai H. A small physiological electric field mediated responses of extravillous trophoblasts derived from HTR8/SVneo cells: involvement of activation of focal adhesion kinase signaling. PLoS One 2014;18:e92252.
22. Pinco KA, He W, Yang JT. alpha4beta1 integrin regulates lamellipodia protrusion via a focal complex/focal adhesion-independent mechanism. Mol Biol Cell. 2002.13:3203-17.
23. C M Illingworth, A T Barker. Measurement of electrical currents emerging during the regeneration of amputated finger tips in children. Clin Phys Physiol Meas. 1980.1:87-89.

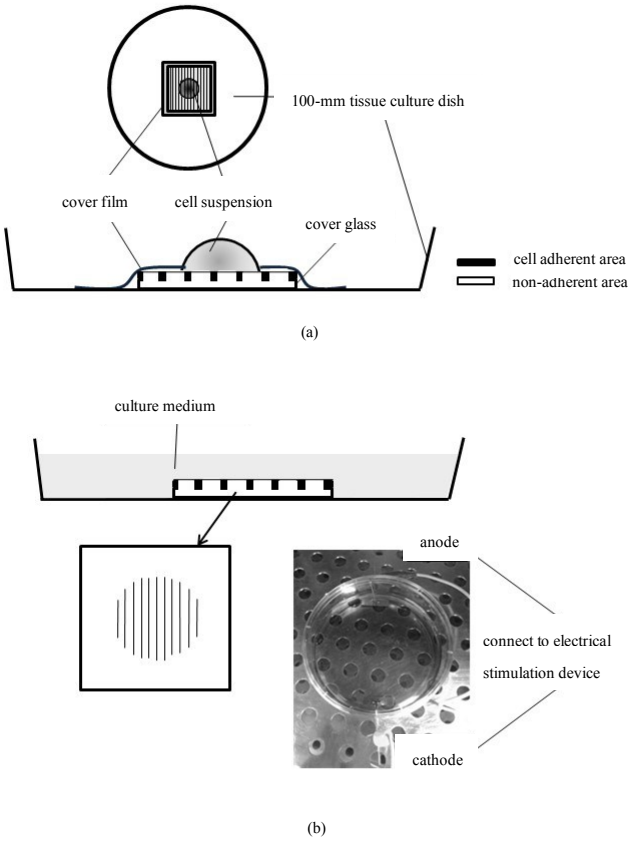

Figure. 1

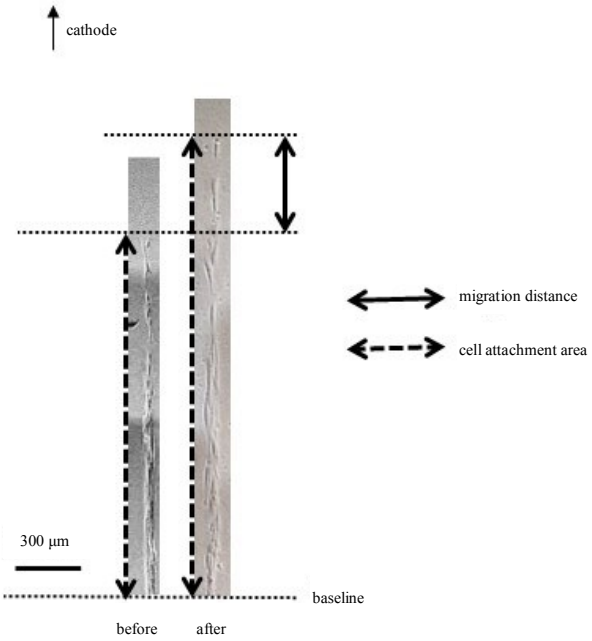

Figure. 2

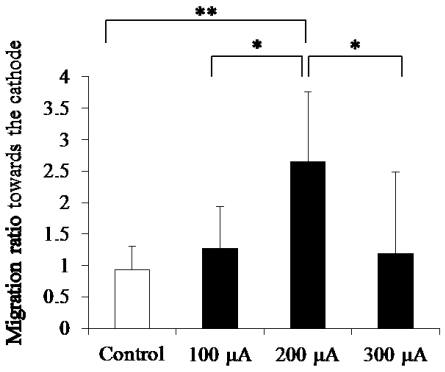

Figure. 3

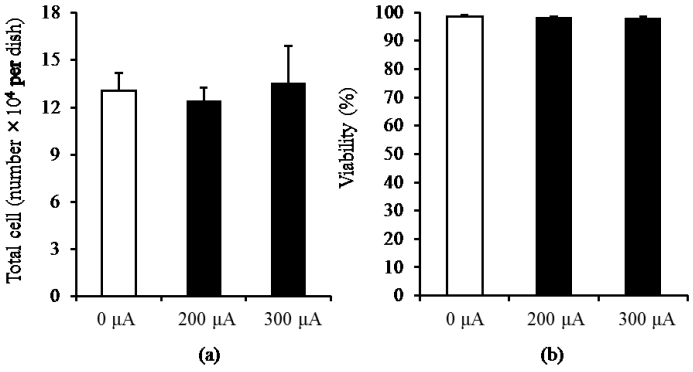

Figure. 4

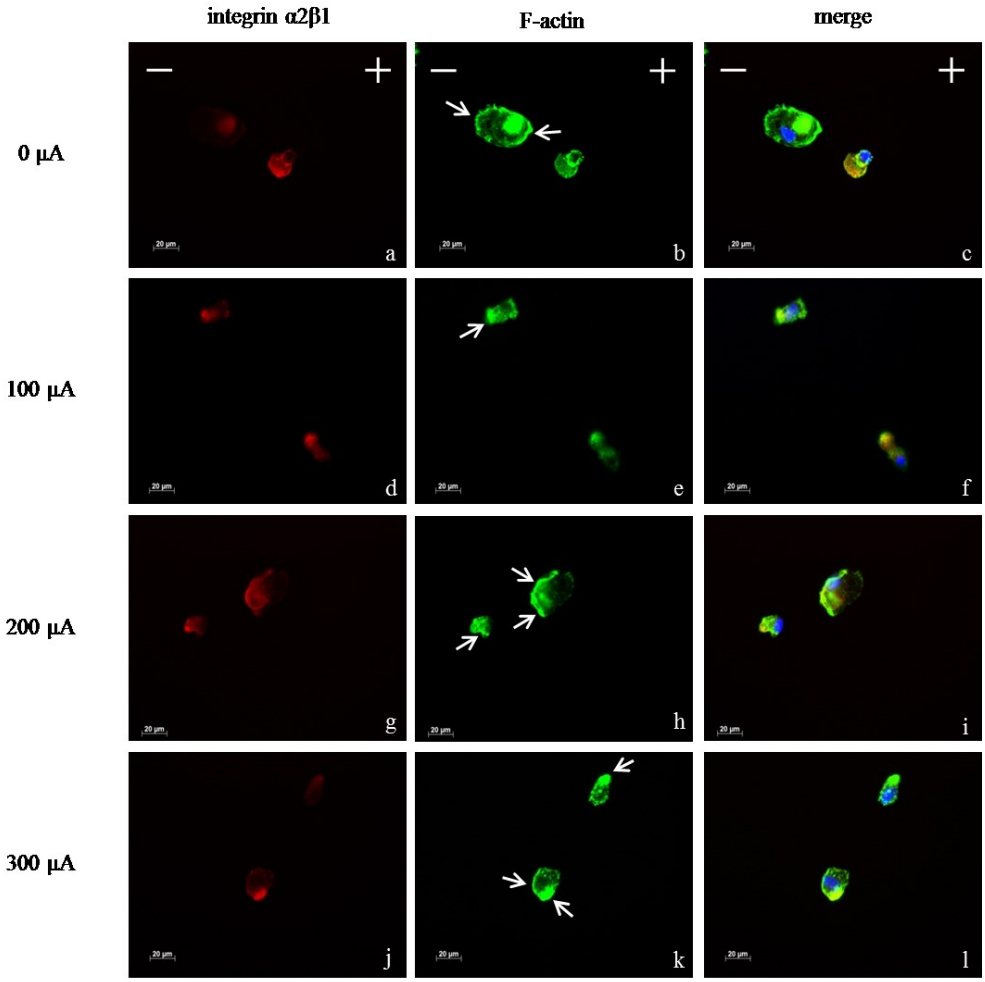

Figure. 5

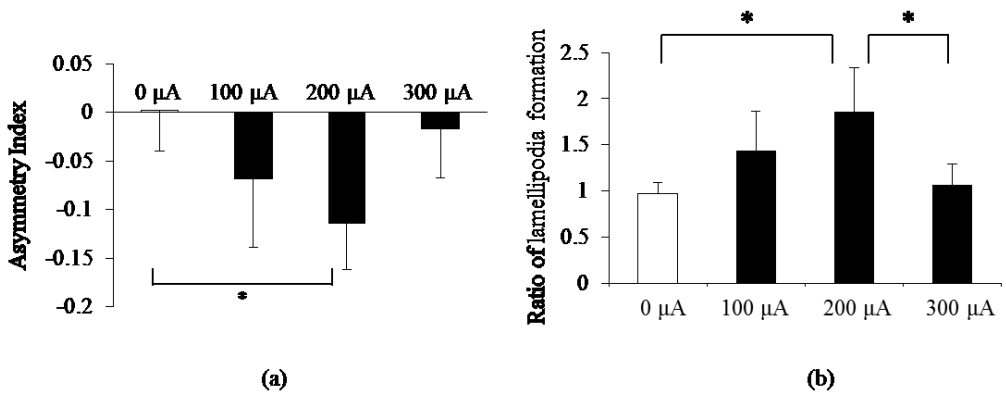

Figure. 6

Figure Legends

Figure 1. Schema of procedure of cell seeding and MPMC stimnulation
(a) Cell seeding
(b) MPMC stimulation

Figure 2. The migration distance in 200 μA group
Representative migration of the groove was indicated by phase-contrast microscopy. Magnification: 400x. Scale bar: 300 μm. The distances of cell attachment area before and after MPMC stimulation were evaluated, and these differences was calculated. In MPMC group, fibroblasts migrated towards the cathode.

Figure 3. The ratio of migration length towards cathode to anode
The migration ratio for 0, 100, 200, and 300 μA groups were evaluated (n=8-10). At 200 μA, fibroblast migration towards the cathode was significant ($p<0.01$), but at 300 μA, the migration ratio decreased to less than the ratio at 100 μA. Data was expressed as mean ± S.D. **$p<0.01$ and *$p<0.05$, Tukey-Kramer.

Figure 4. Cell number and cell viability by MPMC stimulation of more than 200 μA
Cell number (a) and cell viability (b) were measured by cell count using trypan blue staining (n=6). There were no significant differences between 0 μA and MPMC groups. Data were expressed as mean ± S.D. and analyzed by Tukey-Kramer.

Figure 5. Integrin α2β1 polarisation and lamellipodia formation in fibroblasts
Immunofluorescence images of integrin α2β1 (red), F-actin (green), and nuclei (blue) staining. Integrin α2β1 was observed over the entire cell surface in 0 μA (a), while integrin α2β1 polarized in cell edges in MPMC groups (d, g, j). Lamellipodia (arrows) was formed in the cathode and anode facing at 0 μA (b). In MPMC groups, lamellipodia formation was observed at cell edge of the cathode or anode (e, h, k). The images of integrin α2β1, F-actin, and nuclei were superimposed (c, f, i, l) with axio vision (Zeiss, Oberkochen, Germany).

Figure 6. Asymmetry index of integrin α2β1 and ratio of lamellipodia formation at cathode edge to anode edge

(a) Integrin α2β1 polarisation was assessed by asymmetry index. Integrin α2β1 was significantly polarized to the cathode at 200 μA (n=4).

(b) The number of lamellipodia formation at the cathode edge and anode edge was counted and calculated the ratio of lamellipodia formation at the cathode to anode. Lamellipodia formation at the cathode edge was significantly higher at 200 μA than 0 and 300 μA (n=4). Data was expressed as mean ± S.D. *$p<0.05$, Tukey-Kramer.