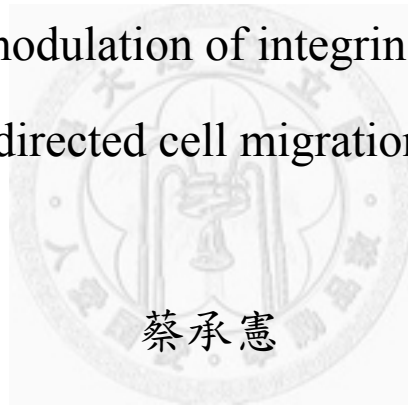國立臺灣大學醫學院暨工學院醫學工程學研究所

碩士論文

Graduate Institute of Biomedical Engineering

College of Medicine and College of Engineering

National Taiwan University

Master Thesis

利用電場調變整合素蛋白極化及引導細胞移動現象

Electric field modulation of integrin polarization and

directed cell migration

蔡承憲

Cheng-Hsien Tsai

指導教授：趙本秀 博士

Advisor: Pen-Hsiu Grace Chao, Ph.D.

中華民國 100 年 8 月

August, 2011

國立臺灣大學碩士學位論文口試委員會審

定書

利用電場調變整合素蛋白極化以及引導細胞移動現象

Electric field modulation of integrin polarization and

directed cell migration

本論文係蔡承憲君(R98548017)在國立臺灣大學醫學工

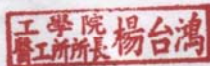程學研究所、所完成之碩士學位論文,於民國100 年07 月18

日承下列考試委員審查通過及口試及格,特此證明

口試委員：

_____

（指導教授）

_____   _____

_____   _____

_____   _____
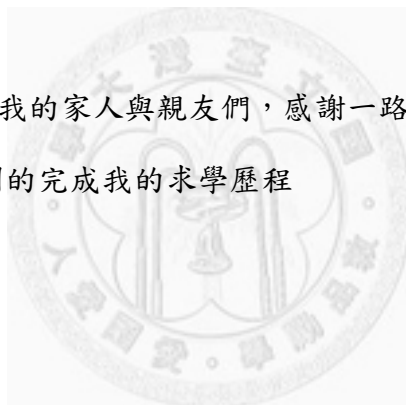
_____   _____

所長 ：   _____

誌謝

　　兩年的學習與實驗終於到了一個段落，在這其中充斥著無數的感激，首先得感謝趙本秀老師平時的教導，在不斷的討論、規劃與不厭其煩的指導中得到了論文的方向，從無到有的做完這篇論文。感謝口試委員林峰輝教授與郭柏齡教授，在百忙之中抽空參加我的口試，並且針對內容提出建議，使得這篇論文更加完備。另外要更加感謝林峰輝教授提供了共軛焦顯微鏡實驗儀器，這大大的幫助我進行論文中的螢光染色顯像的部分。

　　要特別感謝曾經在我實驗中投注心力的仕帆、晏彰、均源，嘉襄、祐甄、沿毓、新民、守謙、向儀、維仁，在實驗的進行上給予協助也讓我順利而且歡樂的完成研究所的學業。

　　最後，感謝永遠支持我的家人與親友們，感謝一路上陪伴自己走過的人，有大家的支持，我才能順利的完成我的求學歷程

中文摘要

電刺激被臨床使用在疼痛的治療以及促進傷口癒合。在骨科的處理上,施予電場可以促進骨頭的癒合以及促進韌帶的復原。在實驗中,我們選用物理治療上時常使用的電刺激波形並檢視對於前十字韌帶纖維母細胞移動以及型態的影響,我們選用的大部分刺激參數都可以增加纖維母細胞的移動,然而對於移動方向上有著十分顯著的差異。此外,前十字韌帶纖維母細胞只有在直流電刺激下會造成細胞有延伸排列的情形。實驗的結果使我們發現細胞移動速度與方向性上有不耦合的情形,這可能是因為不同的機制在進行調控。我們發現整合素蛋白在電場誘發細胞方向性改變的傳導機轉上扮演重要的控制角色,另外也發現整合素蛋白的重新排列也會帶動 RhoA 排列在負極的現象,這表示電場藉由整合素蛋白的不平均分佈調控了下游重要的傳導分子的分布狀態。本篇研究有助於了解物理治療臨床電療在細胞層面的行為影響,以及建立與釐清電場誘導細胞方向性改變的傳導機轉與整合素蛋白的關係。

關鍵字: 電場,移動,整合素蛋白,RhoA

ABSTRACT

Electrical stimulation is clinically used for the treatment of pain and to promote wound healing. In orthopaedic practices, applied electric fields (EFs) promote bone healing and improve lapine ligament repair in vivo. In the current study, several stimulation waveforms used in physical therapy were adapted to examine their effects on anterior cruciate ligament fibroblast (ACLF) migration and morphology. Most of the waveforms we tested resulted in enhanced fibroblast migration, while their effects on migration directionality were noticeably different. Furthermore, ACLFs elongation and alignment were only found in the DC groups. These findings suggest a decoupling of migration speed and directionality, which may arise from disparate mechanisms. We found that integrin acts as a major player of EF-induced directionality. We also found that integrin redistribution mediate the cathodal redistribution of RhoA. This introduces EF mediates one of the major signaling molecules, which is downstream from the integrin asymmetrically, with stronger redistribution on the cathode, is highly significant functionally. Results from this study may benefit our understanding the electro-therapy treatment on cell behavior and the relation between integrin and EF-induced directionality.

Key Words: Electric field, Migration, Integrin, RhoA

CONTENTS

List of figures

Chapter 1   Introduction

Numerous approaches have been proposed to enhance the healing capability of the anterior cruciate ligament (ACL). Electrical stimulation is widely used for the treatment of pain and to promote wound healing [1]. In orthopaedic practices, applied electric fields (EFs) have been used clinically to promote bone healing [2]. EF has also been shown to improve lapine ligament repair in vivo [3]. We have previously demonstrated that applied EF enhanced ligament fibroblast migration and collagen production, depending on the applied EF parameters[4]. Electric stimulation of various types has been found to be an effective treatment technique for soft and hard tissue healing. Besides the long known, low-frequency currents (galvanization, impulse galvanization, diadynamic currents, TENS, faradic current, HVPGS) are increasingly applied today, as they cause patients less discomfort. Low intensity direct current can accelerate the healing rate of both hard and soft tissue injuries [5]. Electrical stimulation of osteogenesis involves the use of faradic waveform to promote the growth of new bone [6]. Faradic stimulation maximizes the restoration of elastic properties of the muscle by encouraging the correct parallel configurational arrangement of the collagen fibres within the cellular matrix [7]. Diadynamic current is a low frequency and biphasic sinusoidal-like waveform. The phase duration (10~20

ms) is longer than faradic current. Clinical usage of diadynamic current (fluctuation current) is for pain [8], analgesia [9], electro-myo stimulation [10], throboembolism (circulating) [11], and suppurating wounds healing [12]. In particular, several recent reports that high-voltage, pulsed galvanic stimulation (HVPGS) significantly increases proliferation, collagen synthesis and healing rate of skin wounds. Similarly, in animal models HVPGS have shown an accelerated rate of full-thickness burns healing [13, 14]. In the current study, several stimulation waveforms used in physical therapy for promoting tissue repair were adapted to examine their effects on ACL fibroblast migration. Here, we focused on clinical electro-therapy on cell migration behavior.
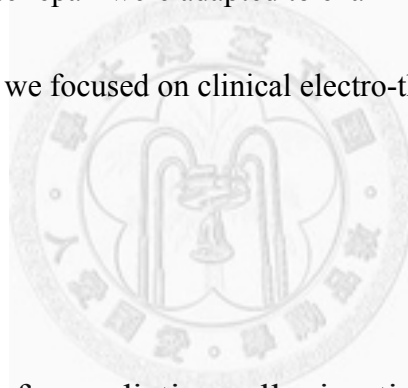
Integrin: important role for mediating cell migration

Cell adhesion underlies several important physiological processes, such as cell migration, spreading, polarity, anchoring, differentiation, morphogenesis and wound healing. Focal adhesions are provided by adhesion molecules and expressed at the cell surface of all nucleated cells, and they mediate extracellular binding to cell and tissue substrates and transmit mechanical docking to the intracellular actomyosin or intermediate filament cytoskeleton.

During cell migration, many classes of membrane receptors fulfill adhesion and

cytoskeletal coupling functions and provide a range of adhesion strength, specificity

and turn-over rates. High affinity adhesion to ECM ligands is predominantly provided

by the integrin family [15].The integrin-mediated cell substrate interactions and

linkages to the actin cytoskeleton, which is mediating cell movement, and its form

and turn-over dynamics and polarity determine cell speed and directionality.

EF-induced integrin redistribution

The electrocoupling mechanisms are confined to the cell surface. Because cell

membrane receptors such as integrins have been demonstrated to laterally diffuse [16]

and redistributed by ES, the mechanism for altered receptor distribution patterns (e.g.,

clustering) could be offered as at least one of the electrocoupling pathways. In recent

years, laboratories use several ways to change the distribution of integrin to find out

the linkage between integrin redistribution and cell migration. The DC electrical

stimulus (0.1V/cm) facilitates the cellular focal adhesion formation of human

fibroblast in three-dimensional collagen gel by clustering of integrins on the

membrane [17]. Cell migration may be extended to postulate that application of ES

triggers the initial event of integrin clustering [18, 19] that serves as the site for

formation of a focal adhesion by recruiting other molecules such as actin-binding

proteins and signaling molecules, and integrin ligation mediating its activation and/or

the clustering of adhesion components to initiate a nascent adhesion [20, 21].

Members of the Rho Family of GTPases are regulated by

Integrin-mediated Signals

Integrin engagement and subsequent clustering of these receptors in focal adhesions lead to the generation of intracellular macromolecular complexes[22]. Numerous enzymatic activity (GTPases) signaling proteins are also structural components of focal adhesions. The Rho family of GTPases contributes to these integrin-mediated signals; in particular, signals that control cytoskeletal organization involved in changes in cell motility. RhoA are part of the Ras superfamily of proteins that cycle between an active, GTP-bound state and an inactive, GDP-bound state. Activated RhoA is capable of stimulating microfilament bundling in serum-starved cells that are already adherent [23]. Rho is also essential for the formation of focal complexes [24]

Studies devoted to how Ras superfamily protein induces integrin clustering into FAs, and much less are known about the clustering of integrins into focal complexes influences RhoA, Rac and Cdc42. Many studies have also demonstrated that shear-induced RhoA activation is secondary to integrin activation [25]. With

increasing numbers of GEFs being identified [26]Rho can be activated by integrin

ligation [27, 28], leading to the formation of stress fibers and FAs. Many studies have

also indicated that RhoA is activated upon integrin-mediated adhesion [29, 30].

Development in the field has been the recognition that integrin-mediated adhesion

stimulates activation of RhoA. GTP-bound RhoA measurement reveals that

integrin-mediated adhesion causes modest increase in RhoGTP levels [27].

Integrins not only control the activation of Rho family but also separately

regulate the translocation of activated RhoA, Rac1 and Cdc42 to the plasma

membrane [31]. Integrin signals regulate the location of membrane domains such as

lipid rafts and thereby control domain-specific signaling events in

anchorage-dependent cells. RhoA is also thought to be concentrated in lipid rafts [32],

and GTP-Rac1 binds more effectively to membranes from adherent than from

suspended fibroblasts, indicating that integrins regulate RhoA and Rac1 membrane

binding sites at the cell surface [31]

We anticipate that adhesion-mediated regulation of Rho family GTPases will

play an important role in the complex process of cell migration behavior

In this experiment, we look further into the hypothesis that (1) different

waveform (direct current, diadynamic current, faradic current, sinusoidal current, and

high voltage pulsed galvanic stimulation) caused different integrin distribution and

induced different fibroblast cell behavior (cell migration, orientation) (2) Electrical

stimulation waveforms affects integrin redistribution which mediates EF-induced

directionality. (3) integrin clustering and redistribution serves a mediator to activate

and redistribute RhoA then induces cell migration directionality.

Chapter 2   Materials and methods

Cell culture

Anterior cruciate ligament (ACL) was harvested from porcine knee joint and cut into cubes. The explants were cultured in DMEM (Biowest) supplemented with 10% FBS (Sigma), penicillin and streptomycin, until the fibroblasts had grown out to confluence. For electric field studies, cells were released from high density monolayer cultures using a trypsin/EDTA solution and plated at 1.8×10^4 cells / cm^2 on to sterile glass slides. After attachment for 2 hrs in a 5 percent CO_2 incubator at 37°C, the slide was gently rinsed with medium and then positioned in a custom galvanotaxis chamber, which was assembled under a sterile laminar flow hood.

Electric field

A customized electrical stimulation chamber was used as previously described [4] (Figure 1). Constant direct current (DC) EF was applied at either 30 V or 0.19 V across the chamber using a Keithley SourceMeter. Other waveforms that were applied

using a custom stimulator (Dynaprog, MingQuo, Taiwan) as illustrated in Figure 2.

All waveforms and the constant 0.19 V group except for HVPGS were controlled to

have the same amount of total current flow (1.9×10^{-5}As). Faradic current (Far)

represented a graduation series of triangular waves peaking at 15 V at 50 Hz.

High-voltage pulsed galvanic stimulation (HVPGS) consists of monophasic,

twin-spike pulses that have a fixed pulse duration of 100 μs and max intensity at 100

V with a frequency of 2.5 Hz. Sinusoidal waves (sin) have a peak intensity of 1.2 V at

50 Hz and the diadynamic waves (diadyn) are rectified monophasicc sinusoidal waves

Pharmacological treatment

Cholesterol treatment

Cells were preincubated for 30 min with 1 mM ChH (cholesteryl hemisuccinate)

(Cat # C6512, Sigma) dissolved in dimethylsulfoxide and diluted in PBS then washed

[33].

Antibody treatment

Cells were preincubated for 30 min with 2.8 μ g/ml anti–porcine integrin α2β1

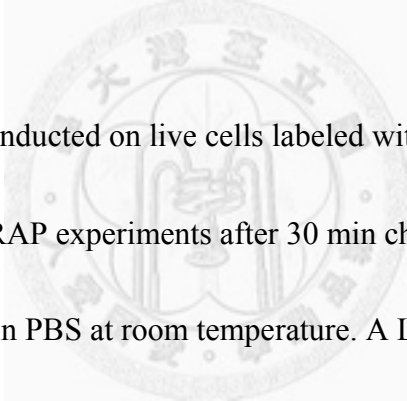antibody (Cat # mab1998, 1 mg/ml) diluted in PBS and washed.

C3 transferase

Cells were preincubated for 2hrs with 2 μ g/ml C3 transferase (Cat # CT04, cytoskeleton) diluted in serum free medium and washed.

PKA inhibitor

Cells were preincubated for 1hr with 10 μ mol/liter KT 5720 (Cat # k3761, sigma) dissolved in methanol and diluted in PBS then washed
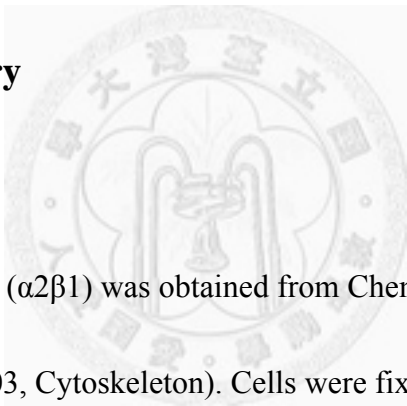
FRAP measurements

FRAP studies were conducted on live cells labeled with DiI (2.5 μ g/ml, sigma). The cells were taken for FRAP experiments after 30 min cholesterol treatment. Measurements were taken in PBS at room temperature. A Leica SP2 confocal microscope was used with a 60X objective for examining the stained cells. After a brief measurement at monitoring intensity, laser pulse (~3s) was used to bleach 50–75% of the fluorescence in the spot. In Fig. 6, the $T_{1/2}$ values were extracted from the averaged FRAP curves, fitting to the analytical expression for a combination of diffusion coefficient D [34, 35].

Migration analysis

Cell migration was analyzed by manually selecting centroid of each cell and tracking them with time. Cell migration distance and angle (θ) were measured and directional velocity, a representation of cell migration in the direction of the field, was calculated by multiplying migration speed with $\cos(\theta)$.

Immunohistochemistry

Anti– integrin antibody ($\alpha2\beta1$) was obtained from Chemicon (mab1998, 1 mg/ml) and RhoA antibody (ARH03, Cytoskeleton). Cells were fixed for 30 min with 4% paraformaldehyde in PBS, and blocked with serum. Cells were incubated overnight in 4^0C with the respective monoclonal antibody diluted in PBS (1:200) supplemented with 1% serum. FITC-conjugated secondary antibodies (AP124F, 2 mg/ml, Chemicon) diluted in 1X PBS (1:200) supplemented with 1% BSA for 1hr was used for labeling the cell surface integrin. An Olympus microscope with a FluoView confocal system was used with a 20X objective for examining and photographing the stained cells. For quantifying the distribution of integrin, we divided the cell into four equal parts and

used image analyzing software (Image J) to measure the fluorescence intensity in the quarter then normalized to the overall cell average. After we measure the normalized intensity into the four regions, the asymetric index (AI) is calculated by subtracting the normalized intensity of the region facing cathode from quarter facing anode.

Statistical analysis

STATISTICA (Stat Soft) was used to perform ANOVA and post-hoc tests (LSD) with $\alpha=0.05$. All bars represent 2-3 separate experiments.
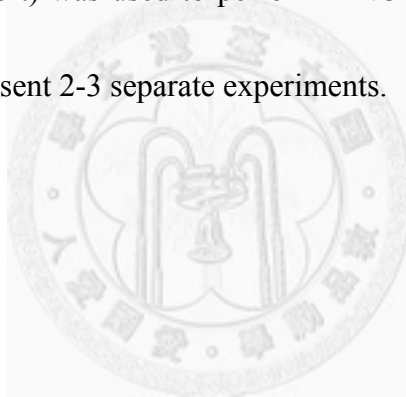
Chapter 3   Results

EF-induced migration

To characterize the disparate waveform-induced cell migration behavior, we applied EFs used in physical therapy to ACLFs plated on glass.

Without an electric field, most fibroblasts migrate in random direction. After 1 hr exposure to DC EF, both 30 V and 0.19V EF caused most cells to elongate perpendicularly to the applied field and migrate toward the cathode. Other groups except for the HVPGS group exhibited enhanced migration speed but did not exhibit any significant elongation or orientation (Figure 4). When compared with the groups subjected to constant DC EFs, all groups were slower except for the diadynamic group (Figure 3A). In terms of directional velocity, which represents the speed toward the cathode, all groups except for the HVPGS group exhibited enhanced migration directionality. When compared with the groups subjected to constant 30V DC EFs, all other groups were slower. Interestingly, no directionality was found with the constant 0.19 V EF group even though it has similarly enhanced migration speed with the 30 V group (Figure 3B).

EF- induced cell surface marker redistribution

To investigate the effects of PT waveforms on integrin redistribution, we measured the fluorescence intensity of the labeled-integrin. Faradic, diadynamic and 30V DC EF redistributed integrin toward cathode, and this observations on EF-induced cell migration seem consistent with studies have also shown that DC electric field can redistribute integrin toward the cathode [17] ,but exposure to HVPGS, 0.19V DC EF, integrin distribute randomly. Other than EF- induced integrin cathodal distribution, sine wave induce integrin move toward anode (Figure 5a). When calculating the normalized fluorescence intensity in the quarter, Faradic, diadynamic and 30V DC EF parameters affect integrin staining stronger in the cathodal quarter than anode quarter but HVPGS causes integrin distribute randomly and sine wave affects integrin move toward opposite side (Figure 5b). After analyzing the asymetric index, we found out the asymmetric value between groups are related to EF-induced directionality (Figure 5c).

Integrin distribution mediate EF-induced directionality

To characterize the effects of the integrin distribution on cell migration behavior,

we investigated cell migration speed and directional velocity for changes induced by

cholesterol hemisuccinate and integrin monoclonal antibody. To reduce membrane

fluidity, we used cholesterol hemissuciate and analyzed membrane diffusion

coefficient quantitatively and indicated that the rate of diffusion and fluorescence

recovery intensity loss by cholesterol embedding (Figure. 6b, 6c). Our observations

on membrane fluidity reduced by cholesterol (Figure. 6a) seem consistent with study

[33] found cholesterol induced random distribution of membrane receptor, manifested

by decreasing membrane fluidity and diffusion rate. Moreover, we found that

randomly distributed integrin in electric field by cholesterol (Figure 7a) also

decreased the EF-induced migration directionality (Figure 7c) but did not affect speed

(Figure 7b). However, the ChH treatment is the non-specific way to reduce the

integrin redistribution, we further blocked integrin function specifically by

anti-integrin antibody, and the function loss of integrin also had same effect on

directionality and speed (Figure 7b, 7c) by cholesterol even though it has asymmetric

distribution (Figure 7a). Based on EF-induced cell migration would be an early and

proximal event after EF onset, we observed the short-term EF exposure to integrin

redistribution. We found out not only 1 hr DC EF exposure can affect integrin

redistribution, in short-term EF expose; both 5 min and 10 min period also affected

integrin redistribute toward cathode (Figure 8) then mediated the cell migration

directionality.

EF- induced RhoA redistribution

We also investigated RhoA distribution tried to find out the correlation with integrin distribution. For 1hr DC EF, the RhoA redistributed toward cathode. Other than EF- induced RhoA cathodal distribution, sine wave induced RhoA move toward anode (Figure 9). The trend of EF-induced RhoA redistribution coincides with integrin (Figure 9). Although exposure to 30V DC EF, the RhoA redistributed toward cathode, but anti-integrin antibody treatment blocked RhoA redistribution phenomenon and affected RhoA distribute randomly (Figure 10).

RhoA staining revealed a lack of RhoA redistribution with functional blocking of integrins, suggesting inhibited intracellular polarization, but inhibition of PKA activity seems does not affect EF-induced RhoA redistribution.

RhoA activity mediate EF-induced directionality

To characterize the effects of the RhoA activity on cell migration behavior, we inhibited RhoA activation by C3 transferanse, and found out similar effect on cell migration in applied EF with integrin blocking and PKA inhibition (Figure 11).

Chapter 4   Discussion

Electric stimulation in physical therapy has been used for many years in repairing non-healing soft and hard tissue. DC mode ES can redistribute cell membrane receptors such as integrin via electrophoresis and electroosmosis mechanism[16] even in the 3-D environment [17]. The EF-induced integrin redistribution serves as the site for formation of a focal adhesion by recruiting other molecules such as actin-binding proteins and signaling molecules[19] and influences the cell migration directionality.

Based on these proposed mechanisms, we first hypothesize that different waveform (direct current, diadynamic current, faradic current, sinusoidal current, and high voltage pulsed galvanic stimulation) caused different integrin distribution and induced different fibroblast cell behavior (cell migration, orientation). Therefore, we investigated the effects of physical therapy electrical stimulation on ligament cell migration and morphology. Most of the waveforms we tested resulted in enhanced fibroblast migration speed, while their effects on cell migration directionality were noticeably different. As we have previously shown, bovine ACL fibroblast and chondrocytes both exhibit different EF threshold response in motility and

directionality[4, 5]. Furthermore, ACL fibroblast exhibit frequency dependent

migration behaviors [4]. Using different waveform and field strengths, we discovered a

decoupling of cell shape and directionality, which may suggest disparate mechanisms

in the two responses [36]. Faradic ad diadynamic stimulation promoted the most

significant response in both speed and directionality. Faradic stimulation has been

shown to promote collagen organization while diadynamic stimulation promotes skin

wound healing [7, 12]. Interestingly, while HVPGS has been demonstrated to increase

proliferation, collagen synthesis and skin wound healing, no significant effect of

increased cell migration is shown here [37].

In terms of cell migration behavior under different field strengths DC EF,

interestingly, no directionality was found with the constant 0.19 V EF group even

though it has similarly enhanced migration speed with the 30 V group. In here, we

hypothesize disparate mechanism control migrate speed and directionality. Zhao et al.

[38] demonstrated using pharmacological inhibitors to find that directionality and

migration rate are regulated by separable pathways. To determine the effects of the

electric waveforms on cell migration behavior and the major factors control migration

mechanism, we investigated EF-induced integrin redistribution.

When analyzing the asymetric index, we found out the asymmetric value

between groups are related to EF-induced directionality. We further look into the

cause and effect between integrin redistribution and EF-induced directionality to clarify the signaling mechanism.

We investigated the time dependent redistribution effectiveness firstly. Asymmetric distribution of integrin receptors labeled with specific antibodies was evident as early as 5 min after EF onset. Integrin redistribution occurs within minutes, which is the early and proximal event like EF-induced cell directionality. Furthermore, we decreased cell membrane fluidity by cholesterol hemisuccinate to reduce EF-induced integrin redistribution. Membrane lipid fluidity depends mainly on the cholesterol content as well as on the degree of saturation of phospholipid acyl chains and phospholipid polar head group composition [39, 40] . Therefore we modify the physical state of membrane lipid matrix (i.e., the membrane lipid fluidity or microviscosity) by alteration of the cholesterol content. The fluorescence recovery after photobleaching (FRAP) experiment shows ChH-treated cell membrane diffusion coefficient is lower than the normal cell membrane. Moreover the ChH-treated membrane eliminated polarization of integrin in the EF, and decreased EF-induced directionality, but did not affect the migration speed. Since the ChH treatment is the non-specific way to reduce the integrin redistribution, we further blocked integrin function specifically by anti-integrin antibody. The Ab-treated cell also decreased EF-induced directionality but did not affect speed; its effect on migration behavior is

18

same as ChH-treated cell. When using confocal microscopy to measure EF-induced

integrin distribution, we found that integrin also redistributed toward cathode. Even

though applied EF polarized the antibody-bound integrin, the functional blocking

treatment abolished the directional response. Taken together, our results indicate that

activation and polarization of α2β1 integrin mediates EF-induced directionality.

EF not only directs cathodal redistribution of integrin but up-regulates the

integrin receptor [30]. Numerous steps have been taken toward understanding the Rho

GTPases during cell polarization and directional migration, and Rho GTPases have

emerged as key regulators in cell migration directionality [43].This introduces EF

activates one of the major signaling molecules, which is downstream factor of

mediating migration from the integrin asymmetrically.

Our current data show that this occurs in parallel with a cathodal redistribution of

RhoA. We report on the involvement of α2β1 integrin mediated RhoA polarization in

EF-induced migration. α2β1 integrin specifically binds to the main ligament matrix

component, type I collagen, and α2 integrin has been shown to modulate ligament

fibroblast migration. By functionally blocking α2β1 integrin with its antibody, we

inhibited directionality without changing motility. This response coincides with the

lack of RhoA polarization. Inhibition of RhoA also abolishes EF-induced

directionality.

The mechanism underlying the key role between RhoA and integrin

redistribution is not clearly understood. One likely possibility role on redistributing

and activating RhoA is PKA. RhoA is a PKA substrate [41] and PKA activity at the

leading edge exhibited a close temporal and spatial correlation with the formation of

activation of RhoA and cell protrusions [42]. We report on the inhibition of PKA

activity by inhibitor also diminished EF-induced directionality, but EF-induced RhoA

polarization still existed. Even though applied EF polarized the RhoA, the PKA

inhibitor treatment abolished the directional response.

In summary, we have shown that EF stimulates the formation of adhesion

molecules redistribution during cell migration, and cell migration directionality is

dependent on integrin and RhoA polarization. Moreover RhoA redistribution is

dependent on integrin-mediated signals. These findings have contributed to the

understanding of some of the modes of action of EF in cell migration directionality.

Finally, our studies additionally identified waveform-induced disparate migration

speed and directionality, and signaling molecules polarization under EF that will be

mediators for EF-induced directionality. Results from this study may benefit our

understanding the electro-therapy treatment on cell behavior and provide further
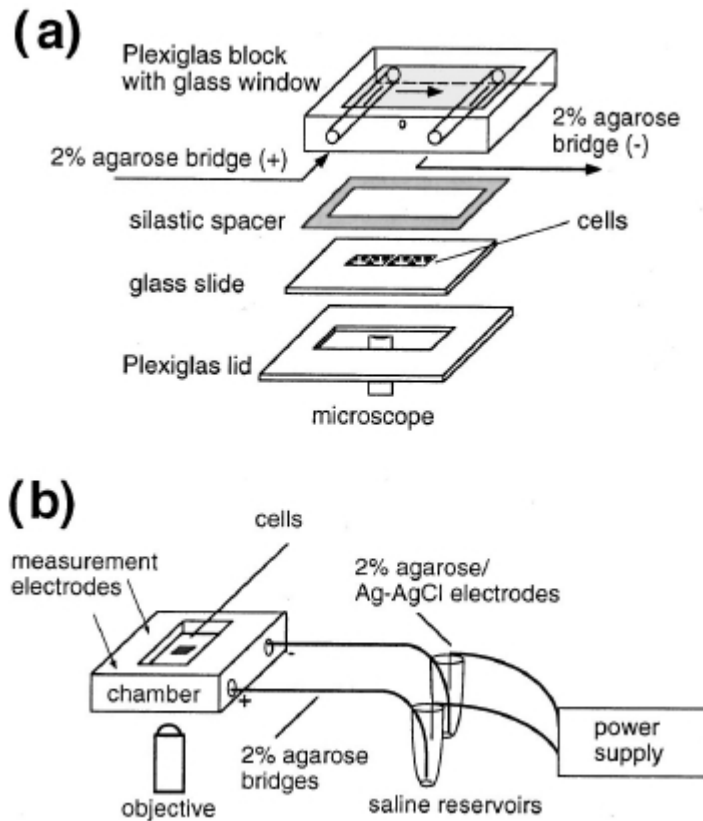
treatment options.

Figure 1 The composition of a galvanotaxis chamber. (a) Cells are put on the center of glass slide and then fit into the chamber. The transparent chamber permits direct observation for cell behaviors with a microscope. (b) The chamber is connected to saline reservoirs with 2% agarose salt bridge. We establish stable electric field across the chamber with external power supply. (Adaped from Chao PH, Roy R, Mauck RL, Liu W, Valhmu WB, & Hung CT (2000) J Biomech Eng 122, 261-267.)
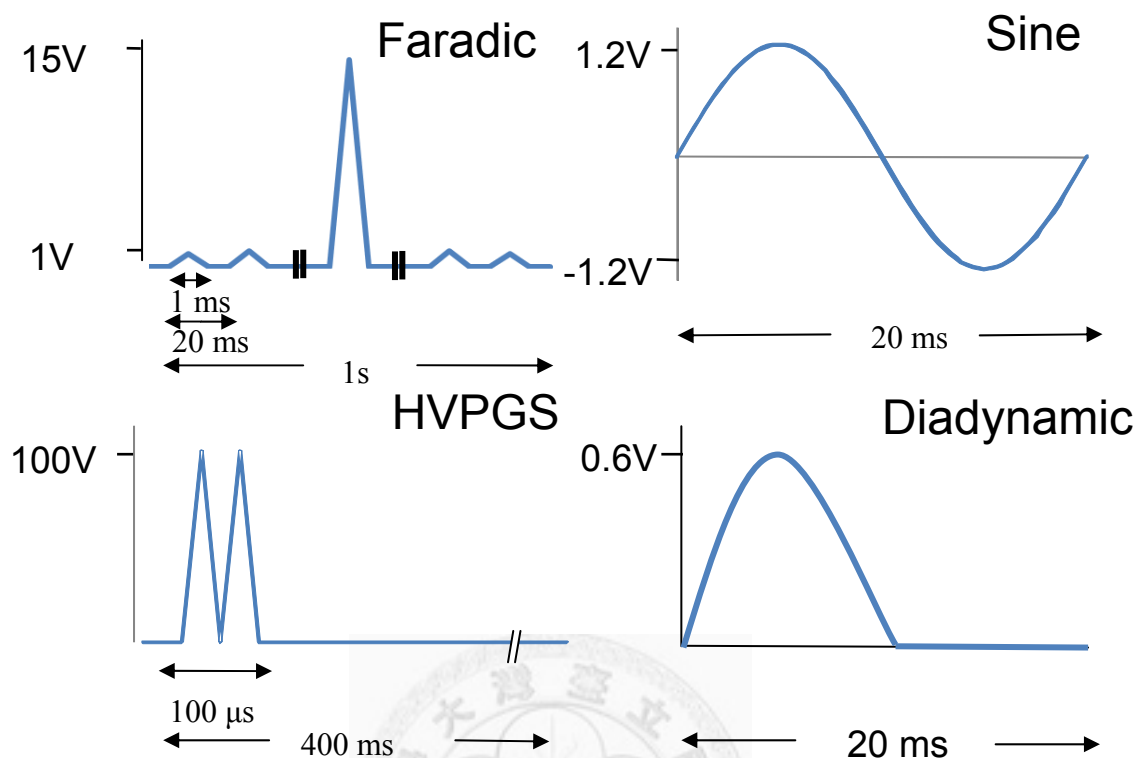
Figure 2 Constant direct current (DC) EF was applied at either 30 V or 0.19 V

across the chamber using a Keithley SourceMeter. Other waveforms that were applied

using a custom stimulator (Dynaprog, MingQuo, Taiwan) as illustrated in Figure 2.

All waveforms and the constant 0.19 V group except for HVPGS were controlled to

have the same amount of total current flow (1.9×10^{-5}As). Faradic current (Far)

represented a graduation series of triangular waves peaking at 15 V at 50 Hz.

High-voltage pulsed galvanic stimulation (HVPGS) consists of monophasic,

twin-spike pulses that have a fixed pulse duration of 100 μs and max intensity at 100

V with a frequency of 2.5 Hz. Sinusoidal waves (sin) have a peak intensity of 1.2 V at

50 Hz and the diadynamic waves (diadyn) are rectified monophasicc sinusoidal waves

Speed

Figure 3A Under electrical stimulation, ACL fibroblasts exhibited significantly different responses in migration. All groups except for the HVPGS group exhibited enhanced migration speed. When compared with the groups subjected to constant DC EFs, all the other groups were slower except for the diadynamic group (* means $p<0.05$ vs 0V control, § means $p<0.05$ vs. 30 V group, n=53-99 cells).
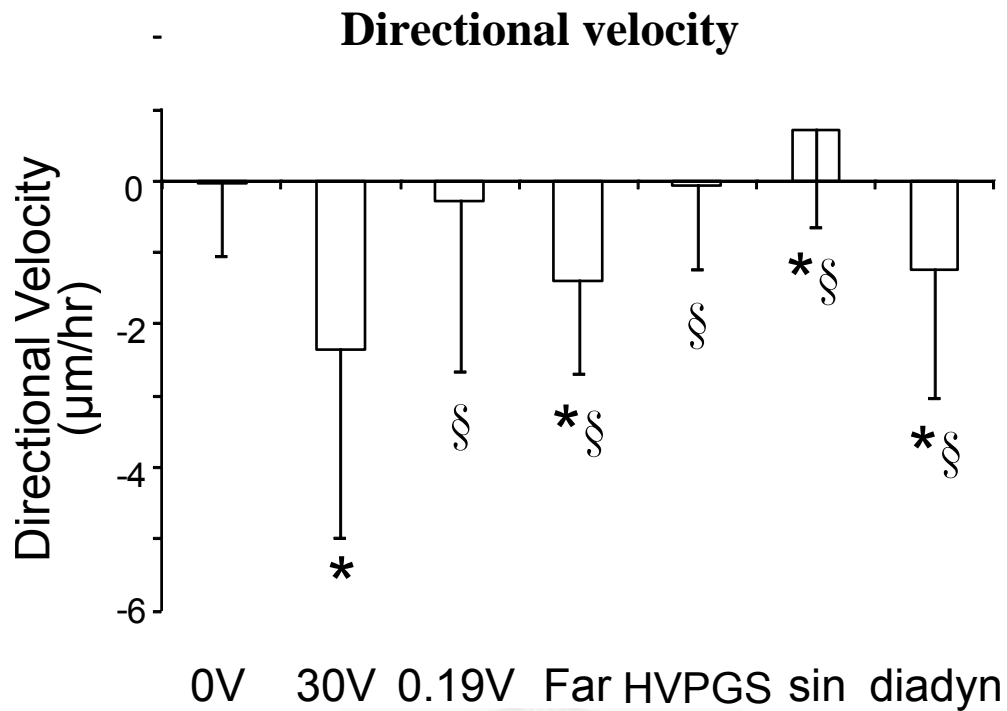
Directional velocity

Figure 3B In terms of directional velocity, all groups except for the HVPGS group exhibited enhanced migration directional velocity. When compared with the groups subjected to constant 30V DC EFs, all the other groups were slower   (* means $p<0.05$ vs 0V control, § means $p<0.05$ vs. 30 V group, n=53-99 cells). Interestingly, no directionality was found with the constant 0.19 V EF group even though it has similarly enhanced migration speed with the 30 V group.
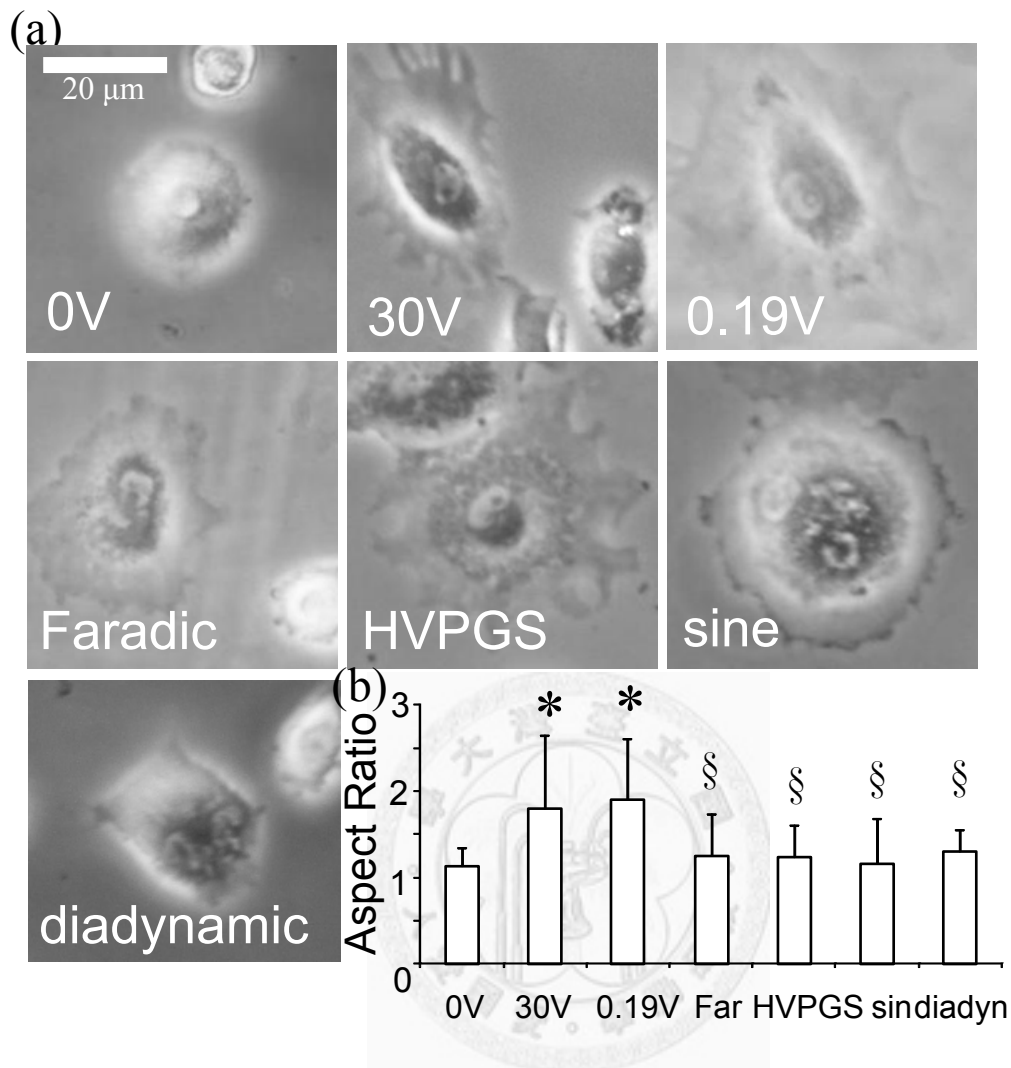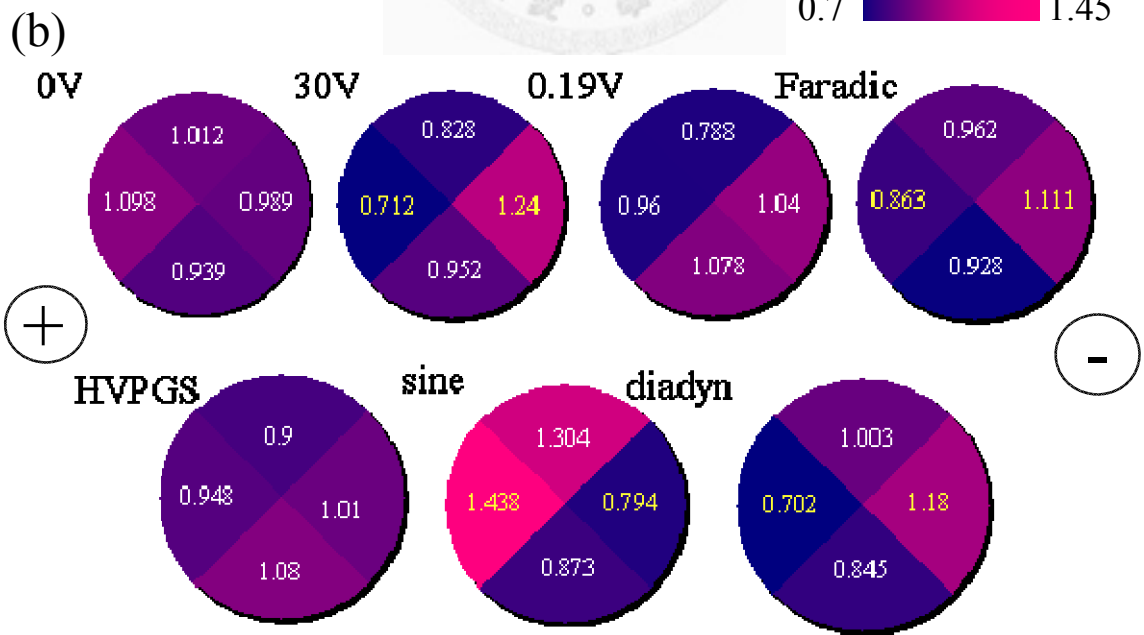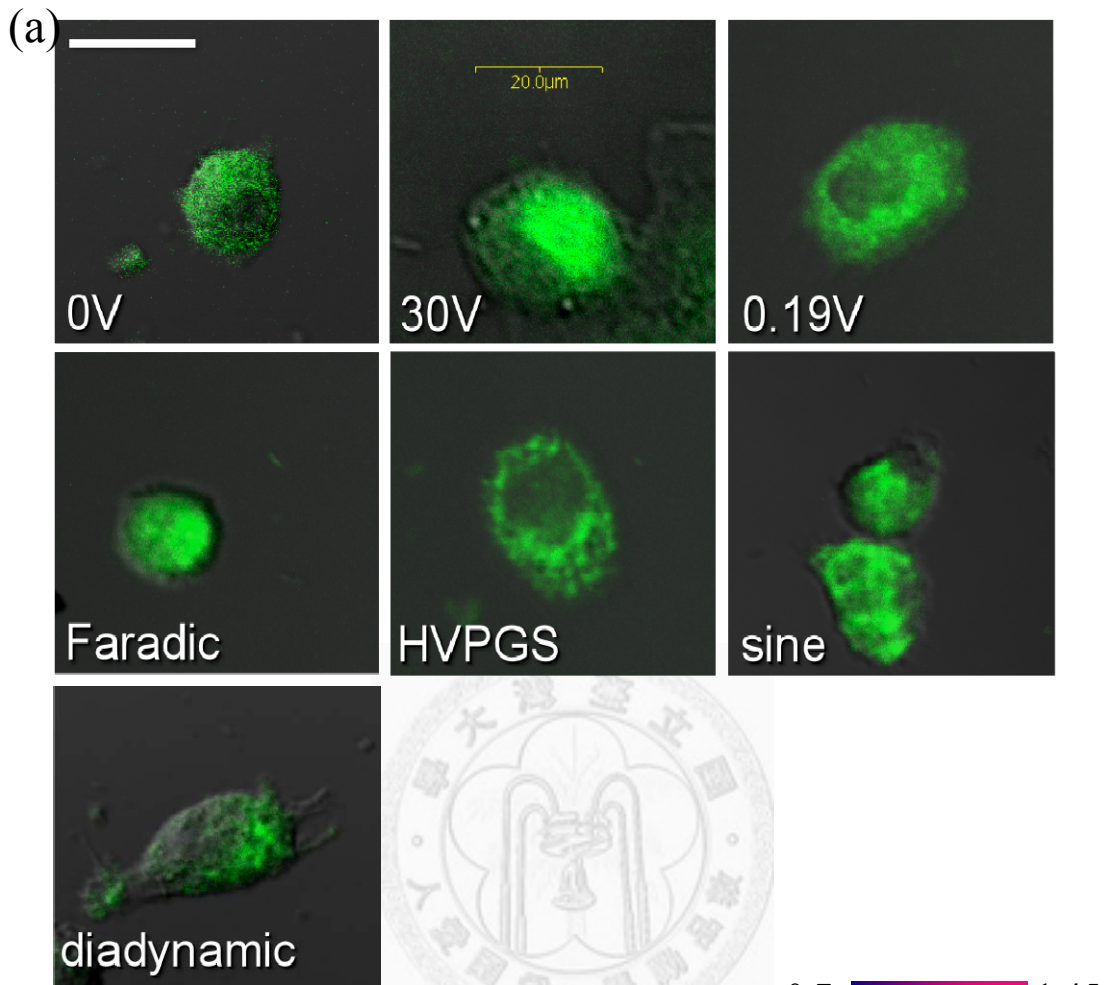
Figure 4 Morphological examination revealed differential cell shape responses.

In the constant 30 V group, ACL fibroblasts exhibited significant elongation and

aligned perpendicular to the constant applied EF groups. All other groups did not

exhibit any significant elongation or orientation (*p<0.05 vs. the 0V control, §p<0.05

vs. the 30 V group, n= 41-156 cells)

(a)

0V

30V
20.0μm

0.19V

Faradic

HVPGS

sine

diadynamic

0.7 ▮ 1.45

(b)

0V

1.012
1.098   0.989
0.939

30V

0.828
0.712   1.24
0.952

0.19V

0.788
0.96   1.04
1.078

Faradic

0.962
0.863   1.111
0.928

(+)

(-)

HVPGS

0.9
0.948   1.01
1.08

sine

1.304
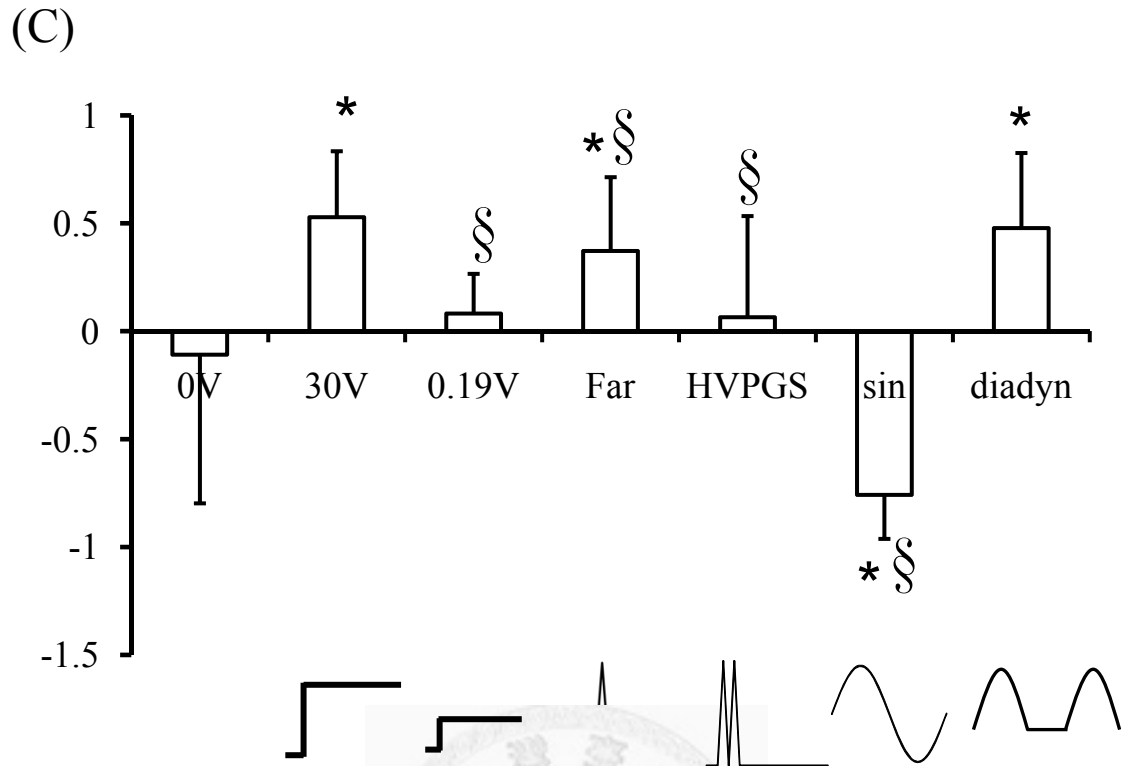1.438   0.794
0.873

diadyn

1.003
0.702   1.18
0.845

Figure 5 Under electrical field, most of waveforms redistributed integrin toward

cathode except for HVPGS, 0.19V DC EF and sine wave. Sine wave induce integrin

move toward anode. (a) immunostaining for integrin α2β1 (b) normalized integrin

distribution (c) asymmetric Index (AI: I cathode- I anode). *p<0.05 vs. the 0V control,
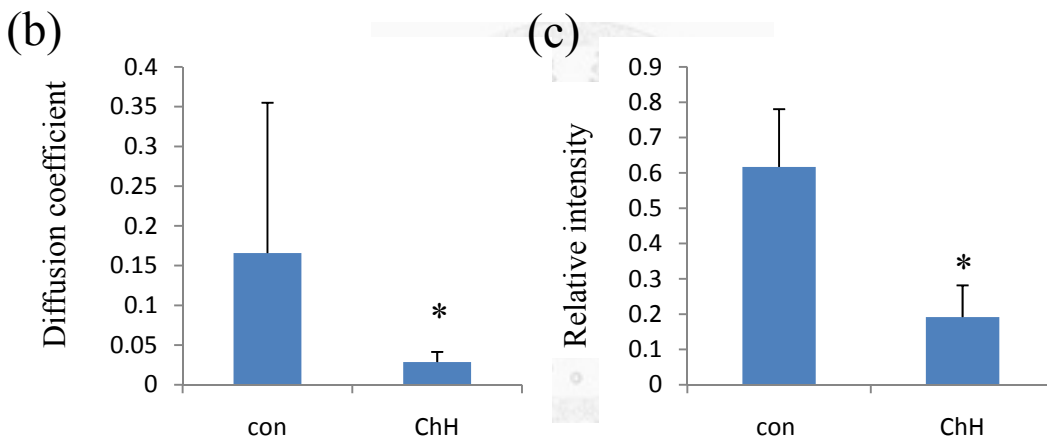
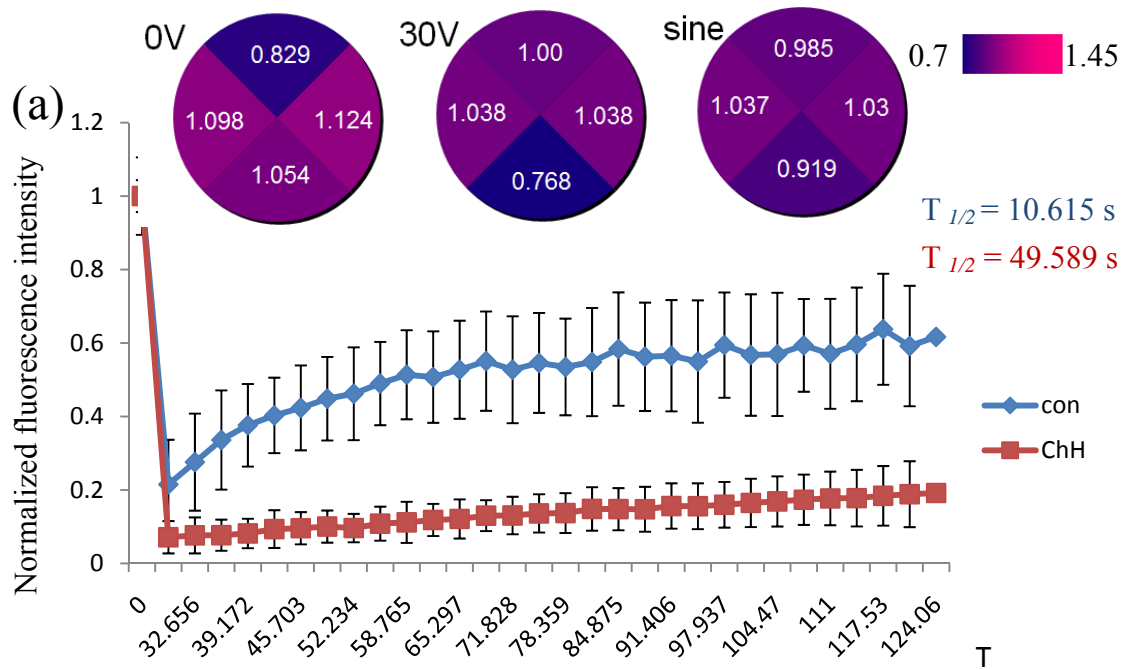§p<0.05 vs. the 30 V group, n= 8-30 cells

Figure 6 effect of cholesterol treatment on FRAP in membrane. Typical FRAP curves (a) of DiI labeled membrane, with (red) or without cholesterol hemisuccinate (blue). Average $T_{1/2}$ values are shown (n= 15–20). Mean ± s.e.m. (n= 15–20) of the diffusion coefficient (D= A/ $4T_{1/2}$) are shown on the lower-left panels (*P< 0.05). Cholesterol induced a decrease in the diffusion rates and fluorescence recovery intensity, accompanied by lower diffusion coefficient (b) and relative intensity (c).
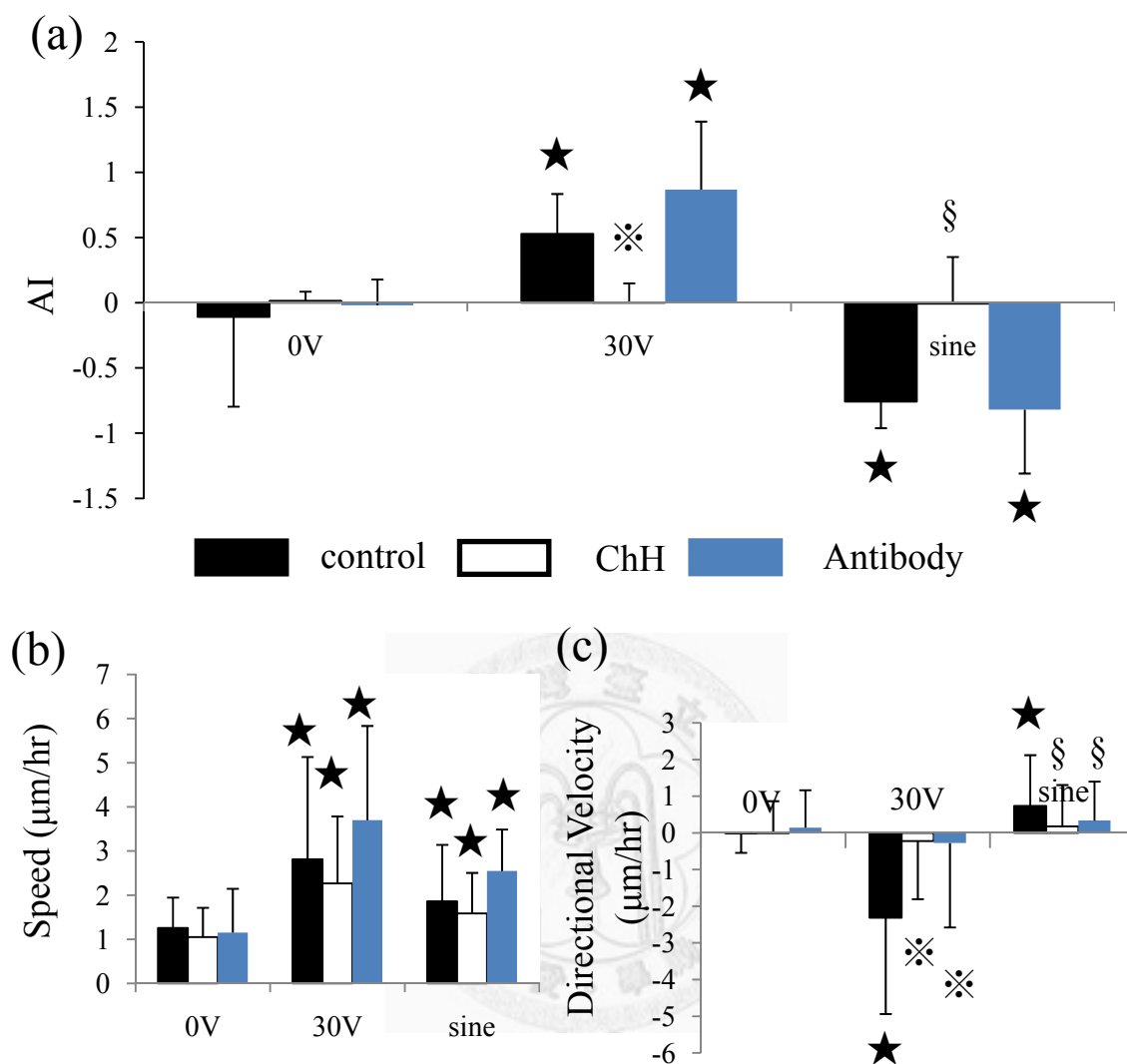
Figure 7 In 30V and sine group, both control and integrin antibody treatment can

induce integrin redistribution, but cholesterol treatment cause integrin distribute

randomly (a) (★p<0.05 vs. 0V, ※p<0.05 vs. the 30V control, §p<0.05 vs. the sine

control group, n= 8-30 cells). Both ChH and antibody treatment, the migration speed

is significant faster on the 30V DC EF and sine wave group (b), but they slower

EF-induced directionality (c). (★p<0.05 vs. 0V, ※p<0.05 vs. the 30V control,
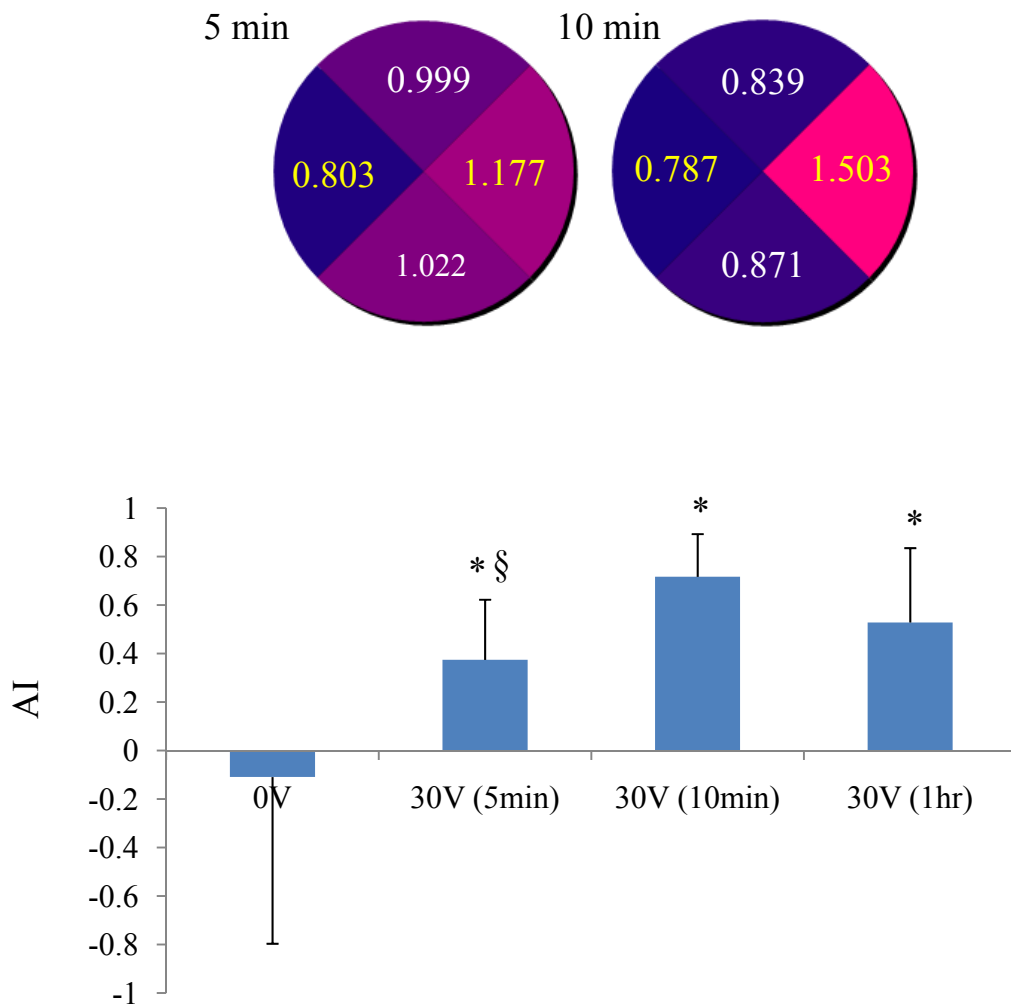
§p<0.05 vs. the sine control group, n= 71-194 cells)

Figure 8 In short-term EF expose, both 5 min and 10 min period affect integrin redistribute toward cathode (* p< 0.05 to 0V control, § p< 0.05 to 10 min period 30V DC EF, n= 15-30 cells).

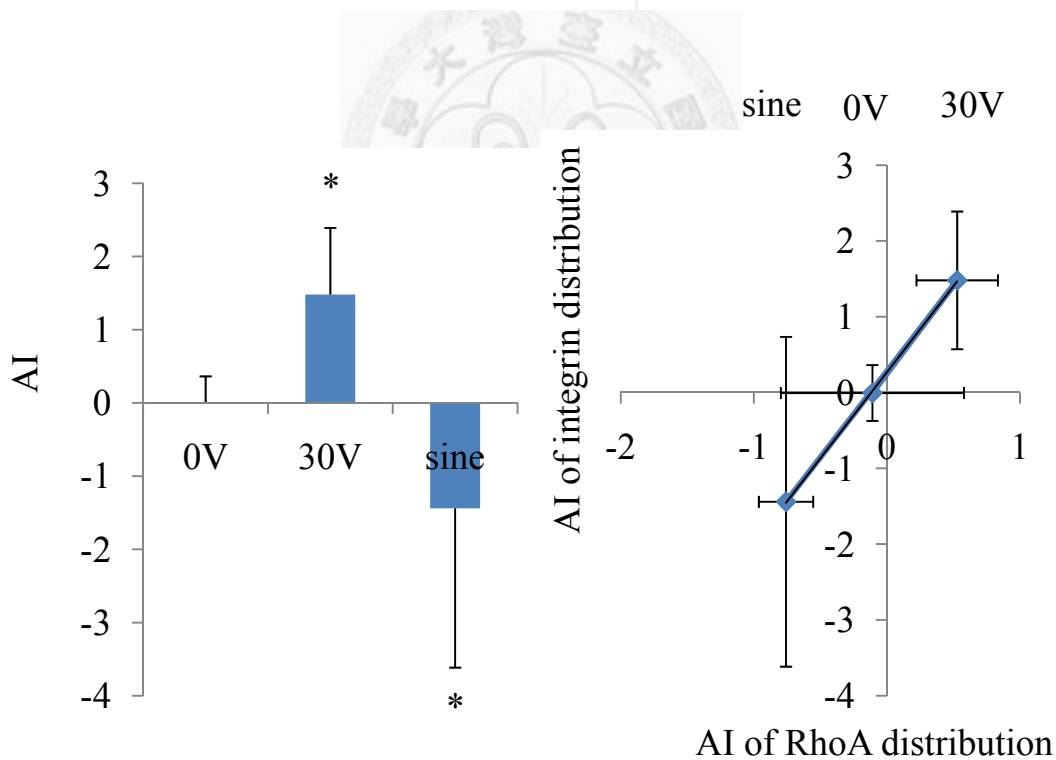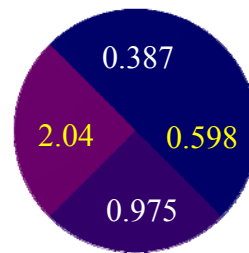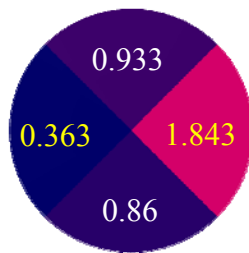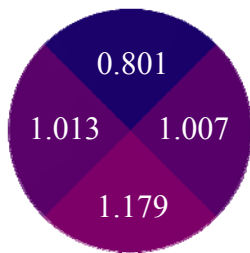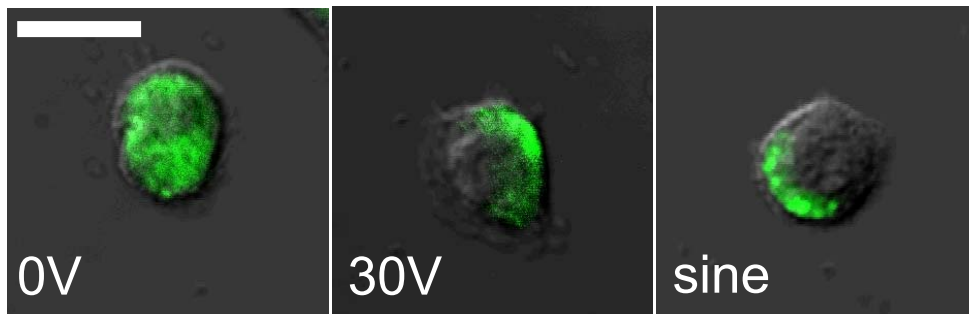**Figure 9** For 1hr DC EF, the RhoA redistributed toward cathode. Other than EF-induced RhoA cathodal distribution, sine wave induce RhoA move toward anode (*means p<0.05 vs. 0V, n= 10-20 cells). According to AI of RhoA and integrin distribution, the EF-induced RhoA redistribution coincides with integrin
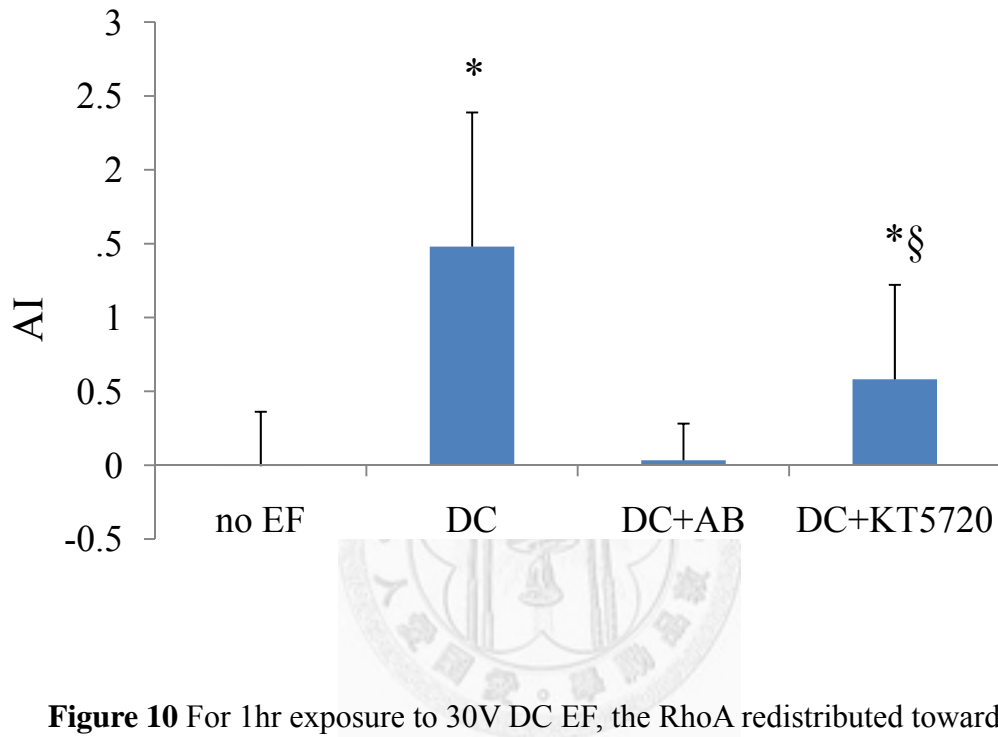
Figure 10 For 1hr exposure to 30V DC EF, the RhoA redistributed toward

cathode, furthermore, inhibition of RhoA activation had a similar effect on RhoA

redistribution in applied EF, but anti-integrin antibody treatment caused RhoA

distribute randomly. (*means $p<0.01$ vs. all other groups, § means $p<0.01$ vs. DC

group, n=10-20 cells)

Figure 11 Inhibition of RhoA and PKA activation by C3 transferase and KT

5720 had similar effect on cell migration. The migration speed is significant faster on

the 30V DC EF (a) but they slower EF-induced directionality (b). (*$p<0.05$ vs. 0V,

§$p<0.05$ vs. other treatment groups, n= 35-118 cells)

Reference

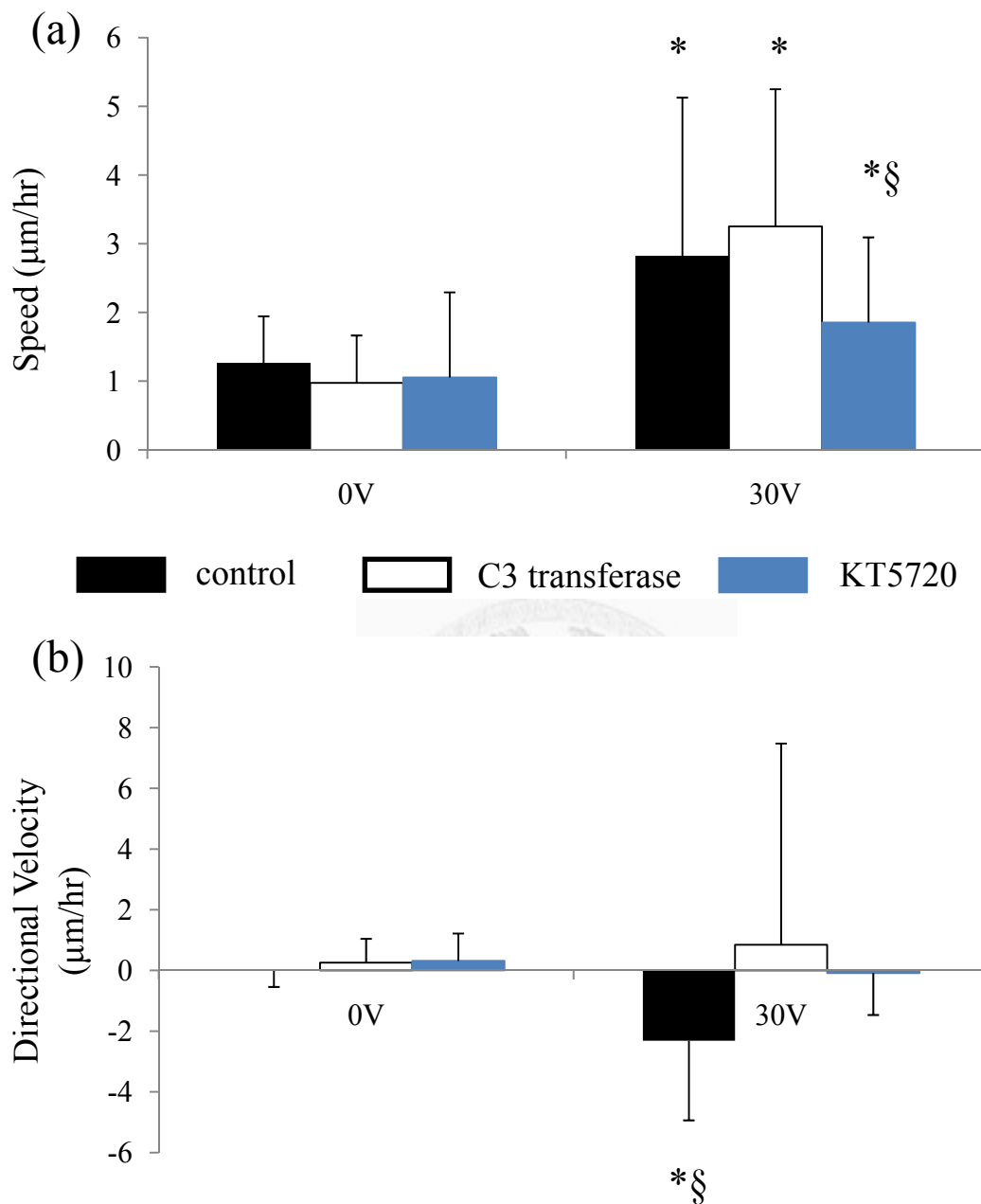1.   Kloth, L.C., *Electrical stimulation for wound healing: a review of evidence from in vitro studies, animal experiments, and clinical trials.* Int J Low Extrem Wounds, 2005. **4**(1): p. 23-44.

2.   Aaron, R.K., D.M. Ciombor, and B.J. Simon, *Treatment of nonunions with electric and electromagnetic fields.* Clin Orthop Relat Res, 2004(419): p. 21-9.

3.   Akai, M., et al., *Electrical stimulation of ligament healing. An experimental study of the patellar ligament of rabbits.* Clin Orthop Relat Res, 1988(235): p. 296-301.

4.   Chao, P.H., et al., *Effects of applied DC electric field on ligament fibroblast migration and wound healing.* Connect Tissue Res, 2007. **48**(4): p. 188-97.

5.   Carley, P.J. and S.F. Wainapel, *Electrotherapy for acceleration of wound healing: low intensity direct current.* Arch Phys Med Rehabil, 1985. **66**(7): p. 443-6.

6.   Campbell, C.E., D.V. Higginbotham, and T.J. Baranowski, Jr., *A constant cathodic potential device for faradic stimulation of osteogenesis.* Med Eng Phys, 1995. **17**(5): p. 337-46.

7.   Ciullo, J.V. and B. Zarins, *Biomechanics of the musculotendinous unit: relation to athletic performance and injury.* Clin Sports Med, 1983. **2**(1): p.

71-86.

8.   Philipson, T., et al., *[The effect of diadynamic current on chronic soft-tissue pain in the neck and shoulder girdle].* Ugeskr Laeger, 1983. **145**(7): p. 479-81.

9.   Lisinski, P., W. Zapalski, and W. Stryla, *[Physical agents for pain management in patients with gonarthrosis].* Ortop Traumatol Rehabil, 2005. **7**(3): p. 317-21.

10.   Romanenko, S.G., O.P. Tokarev, and S. Vasilenko Iu, *[Electrostimulation of laryngeal muscles with fluctuating currents in the treatment of patients with unilateral laryngeal paralysis].* Vestn Otorinolaringol, 2001(3): p. 52-4.

11.   Rusiaev, V.F., Z.N. Salienko, and V.F. Pavlenko, *[Effect of a diadynamic current on the development of thromboembolism].* Patol Fiziol Eksp Ter, 1983(1): p. 10-4.

12.   Dobrova, A.M., et al., *[Use of diadynamic currents to treat suppurating wounds].* Sov Med, 1979(9): p. 55-8.

13.   Brown, M., et al., *High-voltage galvanic stimulation on wound healing in guinea pigs: longer-term effects.* Arch Phys Med Rehabil, 1995. **76**(12): p. 1134-7.

14.   Gogia, P.P., R.R. Marquez, and G.M. Minerbo, *Effects of high voltage galvanic stimulation on wound healing.* Ostomy Wound Manage, 1992. **38**(1):

p. 29-35.

15. Hynes, R.O., *Integrins: versatility, modulation, and signaling in cell adhesion.* Cell, 1992. **69**(1): p. 11-25.

16. Cho, M.R., et al., *Integrin-dependent human macrophage migration induced by oscillatory electrical stimulation.* Ann Biomed Eng, 2000. **28**(3): p. 234-43.

17. Sun, S. and M. Cho, *Human fibroblast migration in three-dimensional collagen gel in response to noninvasive electrical stimulus. II. Identification of electrocoupling molecular mechanisms.* Tissue Eng, 2004. **10**(9-10): p. 1558-65.

18. Cho, M.R., et al., *Induced redistribution of cell surface receptors by alternating current electric fields.* FASEB J, 1994. **8**(10): p. 771-6.

19. Giancotti, F.G. and E. Ruoslahti, *Integrin signaling.* Science, 1999. **285**(5430): p. 1028-32.

20. Calderwood, D.A., S.J. Shattil, and M.H. Ginsberg, *Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling.* J Biol Chem, 2000. **275**(30): p. 22607-10.

21. Carman, C.V. and T.A. Springer, *Integrin avidity regulation: are changes in affinity and conformation underemphasized?* Curr Opin Cell Biol, 2003. **15**(5): p. 547-56.

22. Yamada, K.M. and S. Miyamoto, *Integrin transmembrane signaling and cytoskeletal control.* Curr Opin Cell Biol, 1995. **7**(5): p. 681-9.

23. Ridley, A.J. and A. Hall, *The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.* Cell, 1992. **70**(3): p. 389-99.

24. Hotchin, N.A. and A. Hall, *The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases.* J Cell Biol, 1995. **131**(6 Pt 2): p. 1857-65.

25. Yang, B., et al., *p190 RhoGTPase-activating protein links the beta1 integrin/caveolin-1 mechanosignaling complex to RhoA and actin remodeling.* Arterioscler Thromb Vasc Biol. **31**(2): p. 376-83.

26. Whitehead, I.P., et al., *Dbl family proteins.* Biochim Biophys Acta, 1997. **1332**(1): p. F1-23.

27. Ren, X.D., W.B. Kiosses, and M.A. Schwartz, *Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton.* EMBO J, 1999. **18**(3): p. 578-85.

28. Barry, S.T., et al., *Requirement for Rho in integrin signalling.* Cell Adhes Commun, 1997. **4**(6): p. 387-98.

29. Chong, L.D., et al., *The small GTP-binding protein Rho regulates a*

phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell, 1994.
79(3): p. 507-13.

30.   Clark, E.A., et al., *Integrin-mediated signals regulated by members of the rho family of GTPases.* J Cell Biol, 1998. **142**(2): p. 573-86.

31.   Pozo, D., et al., *Identification of G-protein coupled receptor subunits in normal human dental pulp.* J Endod, 2000. **26**(1): p. 16-9.

32.   Kumanogoh, H., et al., *Biochemical and morphological analysis on the localization of Rac1 in neurons.* Neurosci Res, 2001. **39**(2): p. 189-96.

33.   Carmena, M.J., et al., *Cholesterol modulation of membrane fluidity and VIP receptor/effector system in rat prostatic epithelial cells.* Regul Pept, 1991. **33**(3): p. 287-97.

34.   Axelrod, D., et al., *Mobility measurement by analysis of fluorescence photobleaching recovery kinetics.* Biophys J, 1976. **16**(9): p. 1055-69.

35.   Sprague, B.L., et al., *Analysis of binding reactions by fluorescence recovery after photobleaching.* Biophys J, 2004. **86**(6): p. 3473-95.

36.   McLaughlin, S. and M.M. Poo, *The role of electro-osmosis in the electric-field-induced movement of charged macromolecules on the surfaces of cells.* Biophys J, 1981. **34**(1): p. 85-93.

37.   Bourguignon, G.J. and L.Y. Bourguignon, *Electric stimulation of protein and*

DNA synthesis in human fibroblasts. FASEB J, 1987. **1**(5): p. 398-402.

38. Zhao, M., et al., *Membrane lipids, EGF receptors, and intracellular signals colocalize and are polarized in epithelial cells moving directionally in a physiological electric field.* FASEB J, 2002. **16**(8): p. 857-9.

39. van Ginkel, G., H. van Langen, and Y.K. Levine, *The membrane fluidity concept revisited by polarized fluorescence spectroscopy on different model membranes containing unsaturated lipids and sterols.* Biochimie, 1989. **71**(1): p. 23-32.

40. Yeagle, P.L., *Lipid regulation of cell membrane structure and function.* FASEB J, 1989. **3**(7): p. 1833-42.

41. Lang, P., et al., *Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes.* EMBO J, 1996. **15**(3): p. 510-9.

42. Machacek, M., et al., *Coordination of Rho GTPase activities during cell protrusion.* Nature, 2009. **461**(7260): p. 99-103.

43. Fukata, M., M. Nakagawa, and K. Kaibuchi, *Roles of Rho-family GTPases in cell polarisation and directional migration.* Curr Opin Cell Biol, 2003. **15**(5): p. 590-7.