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Regulation of mesenchymal stem cell adhesion and orientation in 3D collagen scaffold by electrical stimulus

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Abstract

Cell adhesion and orientation are important for both natural and engineered tissues to fully achieve physiologic functions. Based on diverse cellular responses induced by electrical stimulus on 2D substrate, we applied non-invasive electrical stimulus to regulate cell adhesion and orientation of bone marrow-derived mesenchymal stem cells (MSCs) and fibroblasts in a reconstituted 3D collagen-based scaffold. While fibroblasts were induced to reorient perpendicularly in response to direct current electrical stimulus, rat MSCs showed only slight changes in cell reorientation. Multiphoton microscopy revealed that rat MSCs exhibited much stronger 3D adhesion, which appears to resist cell reorientation. Only in response to a large electrical stimulus (e.g., 10 V/cm), collagen fibers around rat MSCs became disconnected and loosely reorganized. In contrast, the collagen fibers surrounding the fibroblasts were entangled in a random network and became preferentially aligned in the direction of the electrical stimulus. When incubated with integrin antibodies, both fibroblasts and rat MSCs failed to respond to electrical stimulus, providing evidence that integrin-dependent molecular mechanisms are involved in 3D cell adhesion and orientation. Elucidation of physical regulation of 3D cell adhesion and orientation may offer a novel approach in controlling cell growth and differentiation and could be useful for stem cell-based therapeutic application and engineering tissue constructs.

Keywords: Mesenchymal stem cells; Multiphoton microscopy; Adhesion; Orientation; Electrical stimulus; Integrin; Laser optical tweezers

1. Introduction

Cell orientation and adhesion are known to affect cell behaviors and functions in both natural and engineered tissues. Cell adherence to substrate plays a key role in morphogenesis and organogenesis, especially for cells expanded in a monolayer culture [1,2]. For example, osteogenic stem cell differentiation is positively influenced by strong adhesion onto surface, and attachment can be reduced onto micro-rough surface, resulting in decreased proliferation and increased differentiation [3]. In tissue engineering cells are typically seeded in a 3D scaffold, which not only provides the anchorage for cell adhesion, but also provides a suitable cellular microenvironment through adhesions that regulates cell differentiation, metabolic activity, and cell–cell signaling [4]. Similar to the role of cell adhesion, cell orientation is essential for maintaining normal tissue properties and functions. Cells in biological tissues are typically orientated and spatially patterned. Oriented cells, for instance, could provide favorable adhesion due to higher density of focal contacts and rearrange the cytoskeletal structures (e.g., actin fibers) [5], and also determine the alignment of collagenous matrix in healing ligaments and tendons that are less organized after injury [6]. Moreover, cell columns reflect the direction of growth, which can be particularly rapid towards a certain preferred direction [7,8]. Significant difference in cell orientation has also been documented in pathophysiology. For instance, cells in the abnormal or wound healing show no or less specific orientation in comparison to those in normal tissue [9,10]. One of the ultimate goals in tissue engineering is therefore to mimic the natural cell shape and orientation in the engineered tissue constructs.

Because regulation of cell adhesion and orientation is important for cell-based therapy and tissue engineering, several techniques, including surface topography modification and use of physical stimuli, have been developed to control

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cell orientation. For example, modifications in biomaterial surface properties such as the peak to valley height of the surface structures have been shown to induce cell orientation [5]. Under periodical uniaxial stretching, cells cultured on 2D substrate or in 3D matrix changed the cell shape and became reoriented along the direction of loading [11]. As an alternate mode of physical stimulus, application of electrical stimulus has a few advantages. It is relatively technically simple, flexible and feasible both in vivo and in vitro for 2D and 3D cultured cells. More importantly, since physiological electrical activities generated in biological processes such as embryonic development and wound healing are known to influence these processes through guiding cell directional movement and alignment, many cell types, including fibroblasts, osteoblasts, myoblasts, and neural crest cells have all been found to be able to reorient in response to electrical stimulus [12–17].

Although induced cell adhesion, migration, and orientation in response to electrical stimulus have been well documented using 2D cultured cells, electrically induced cellular behaviors in the 3D scaffold remain unknown. Not only may cells respond differently on the 2D substrate or in 3D scaffold, the interactions between cells and matrix, often ignored in the studies using 2D cultured cells, are crucial for tissue engineering using 3D cultured cells. Because 3D cultured cells are more representative of native biological tissues, these cell cultures may sustain the phenotypic properties for long-term culture and thus enable us to study changes in both the cells and extracellular matrix (ECM) of the engineered tissue. The aim of this present study is therefore to characterize quantitatively the effects of electrical stimulus on the adhesion and orientation of MSCs seeded in the 3D collagen scaffold and to compare with those obtained using terminally differentiated fibroblasts. In addition to determining cellular morphological changes and reorientation, we also investigated the integrin-dependent electrocoupling mechanisms. It should be noted here that electrical stimulus not only can induce changes in cell morphology, but also influence cell behaviors such as migration, proliferation and differentiation; all of these responses may be coupled mechanistically. Application of electrical stimulus could then offer a novel physical approach for controlling cell growth and differentiation in cell based therapy and engineered tissue constructs by regulating cell adhesion and orientation.

2. Materials and methods

2.1. Cell culture

Bone marrow-derived rat MSCs were kindly provided by Prof. Jeremy Mao (from University of Illinois at Chicago). Rat MSCs and HT1080 fibroblasts were cultured in complete media consisting of Dulbecco's Modified Eagle's medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS), 1% antibiotics/antimycotics (final concentration; penicillin 100 units/ml, streptomycin 100 μg/ml and amphotericin B 0.25 μg/ml). Cells were incubated in a culture flask in 5% CO_2 at 37 °C, and media was changed twice a week. Passages between 3 to 9 were used for all experiments. A set of positive control experiments was performed to differentiate rat MSCs into bone cells by using the osteogenic differentiation media that include dexamethasone, β-glycerophosphate and ascorbic acid. We used the molecular markers such as alkaline phosphatase, calcium nodules, and osteocalcin expression to verify the osteogenic differentiation (data not shown).

2.2. Collagen scaffold and cell seeding

Cell cultures in the collagen gel were prepared in the similar procedure described in detail elsewhere [18]. Briefly, 2.0 mg/ml collagen Type I from rat tail (Sigma, St. Louis, MO) in 0.1 M acetic acid was mixed with the same volume of 0.1 M NaOH and 10% (*v*/*v*) 10× Hanks' balanced salt solution (HBSS), acetic acid (0.1 M) was added dropwise to adjust pH to 7.4. Same volume of cell suspension was added in collagen solution (working cell density of 2×10^5 cells/ml) and, after gentle mixing, a predetermined amount of the mixture containing the cells was taken and placed evenly on glass substrate, and then incubated at 37 °C. This procedure yielded the collagen gel of 200 μm in average thickness. The sample was gelatinized within minutes. Culture media was added after 1 h to allow full gelation. The 3D cell culture was further incubated for 12 h before it was used for experiment.

2.3. Electrical stimulus

Detail of the electrical stimulation chamber, shown in Fig. 1, has been described previously [19–21]. Briefly, direct currents from an amplifier (BOP100, KEPCO, Flushing, NY) were

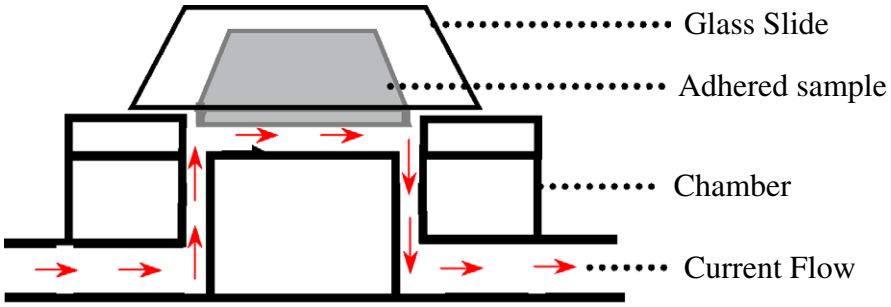

Fig. 1. Schematic drawing of electrical stimulus chamber. Electrical currents from a power supply were applied to the chamber through 2% agar salt bridges to avoid the unwanted byproducts from the metal electrodes and to minimize pH changes.

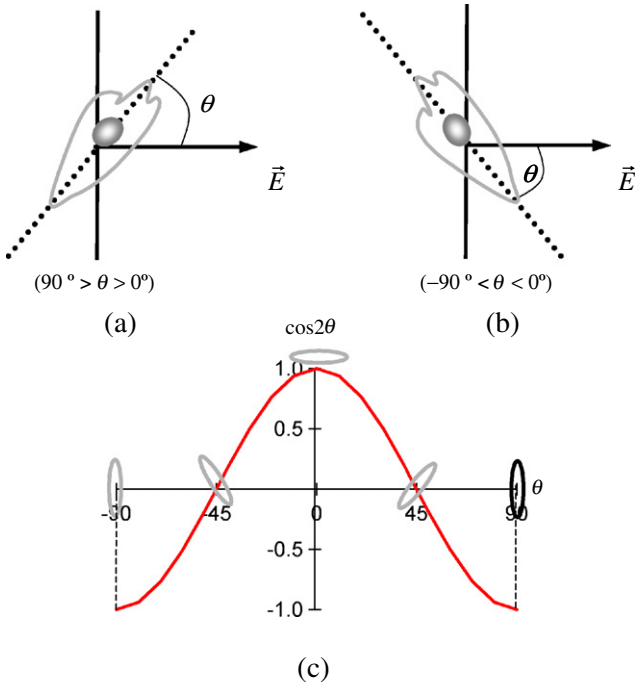

Fig. 2. Illustration of orientation factor (OF) and orientation angle θ. OF, is defined as cos2θ, where θ is the angle between the electric field vector (horizontal axis) and the long axis of the cell. (a) and (b) provide the two cases of θ. Because θ can range between −90° to +90°, the OF lies between the values from −1 to 1. (c) OF as a function of θ. The corresponding cell orientations are schematically shown at specifically chosen OF values.

applied to the chamber through 2% agar salt bridges. Because the cross sectional area of the chamber has been measured with micrometer-precision and the electrical currents delivered to the chamber are precisely controlled by the amplifier, the electrical stimulus strength (accurate within 20%) is computed according to Ohm's law ($J=\sigma E$; J is the current density and σ is the conductivity) and monitored using an oscilloscope. The strength of an electrical stimulus indicates the field intensity (e.g., V/cm). All experiments were performed at room temperature.

2.4. Cell orientation measurements

Fluorescence and differential interference contrast (DIC) images of rat MSCs and fibroblasts in collagen gel were recorded using a Nikon microscope (Eclipse E800). Illuminating light was focused on the sample through a 20×/0.75 NA objective or a 10×/0.25 NA objective and images were recorded using a CCD camera (CoolSnap fx, Roper Scientific, Tucson, AZ). Although multiple layers of cells were observed in the collagen gel, the cells found in the middle layer of the collagen gel were monitored for 60 min at 1 min intervals. 30 to 60 cells were typically observed and tracked in each experiment. Using MetaMorph image processor (Universal Imaging, West Chester, PA), the orientation of each cell was determined by identifying its major axis with respect to the electric field vector. Because the cells were stained with the

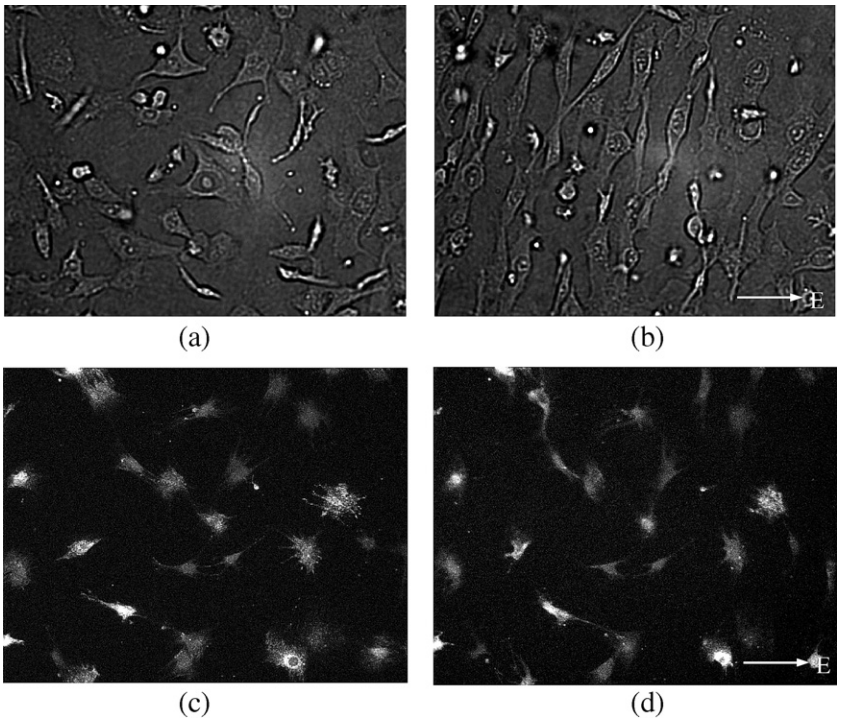

Fig. 3. Cell orientation before and after electrical stimulus. Cells were seeded in the 3D collagen gel. (a) and (b) are DIC images of fibroblasts taken with a 20× microscope objective before and after application of 7 V/cm for 30 min, respectively. (c) and (d) are the fluorescence images of rat MSCs taken with a ×10 microscope objective before and after application of the same electrical stimulus for 30 min. Rat MSCs were imaged using the CellTraker fluorophore for better visualization using a smaller objective. The arrow indicates the direction of electric field vector.

CMTMR cell tracker, the image processor efficiently utilized significant differences in the fluorescence intensity between the CMTMR loaded cells and background to identify cell boundaries and the major and minor axes of the cell. The orientation factor (OF) was calculated from:

$$OF = \langle \cos 2\theta \rangle = 2\langle \cos^2(\theta) \rangle - 1$$

where $\langle \cos^2(\theta) \rangle$ is the average of $\cos^2(\theta)$, and θ is the angle between the direction of electrical field and the long axis of the cell [20,22]. As illustrated in Fig. 2, because the orientation angle of cell can range between −90° to +90°, the OF lies between the values from −1 to 1. A value of zero OF would correspond to random orientation, whereas the values of −1 and 1 would describe perpendicular and parallel orientation of cells, respectively, when measured from the direction of electrical stimulus (e.g., *x*-axis).

3. Results

Fibroblasts and rat MSCs both showed changes in cellular morphology in response to electrical stimulus. Fig. 3 shows images of the cell shape and orientation for these two types of cells in a 3D collagen scaffold before and after exposure to an electrical stimulus. As expected, fibroblasts appeared to contract in the direction of electrical stimulus, followed by cell elongation and reorientation perpendicular to the electrical stimulus. These observations are consistent with the similar findings that have been reported previously [14,23]. In response to a 7 V/cm electrical stimulus, for example, the randomly oriented fibroblasts (Fig. 3a) not only became elongated but also showed clear reorientation (Fig. 3b). In contrast, unlike fibroblasts, most rat MSCs resisted physical changes and maintained the original cell orientation (Fig. 3c) in response to electrical stimulus. Only a small fraction (less than 10%) of rat MSCs became contracted, reoriented, and demonstrated changes in cellular morphology in response to electrical stimulus (Fig. 3d).

Quantitative analysis of the induced cell reorientation in response to electrical stimulus was performed based on the method illustrated in Fig. 2. Fig. 4a shows the time-dependent fibroblast reorientation at varying strengths of electrical stimulus. For example, fibroblasts exposed to a 2 or 4 V/cm did not show significant cell reorientation over a period of 60 min observation (OF∼−0.1). An exposure of cells to a larger electrical stimulus (e.g., 7 V/cm) for 60 min was required to induce a noticeable fibroblast reorientation. In this case, the calculated OF changed from 0.15 (e. g., random) to −0.6,

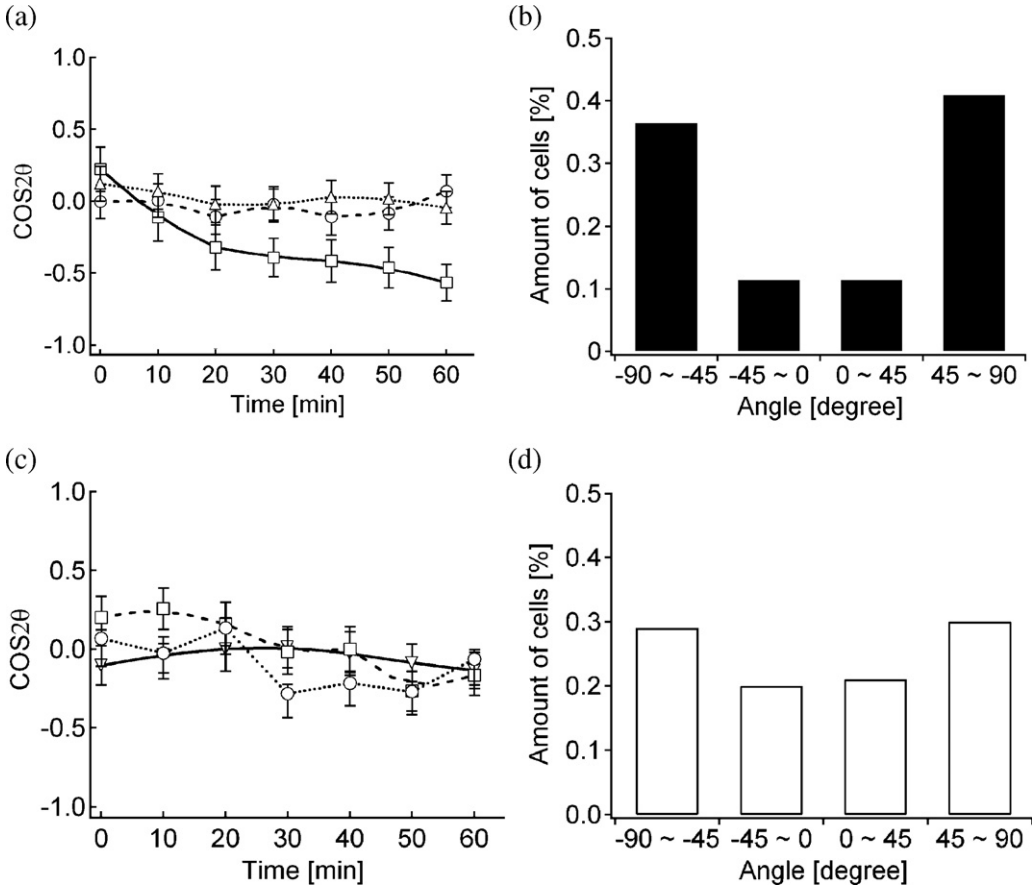

Fig. 4. Time-dependent cell reorientation at varying strengths of electrical stimulus. (a) Fibroblasts in response to 2 (Δ), 4 (O) and 7 V/cm (□). A larger electrical stimulus of 7 V/cm was required to induce a noticeable fibroblast reorientation (e.g. OF=0.6). (b) The number of cells found in the 4 quadrants of each 45° from +90° to −90°. (c) Rat MSCs in response to 4 (O), 7 (□) and 10 V/cm (▽). (d) The number of rat MSCs found in the 4 quadrants was approximately the same. Typically, 30 to 60 cells were observed and tracked in each experiment, and each data point represents the mean±SD from 3 to 6 independent experiments.

indicating that larger electrical stimuli can induce fibroblast reorientation in the 3D collagen scaffold. Following cell reorientation, we counted the number of cells found in the 4 quadrants of each 45° from +90° to −90° (see Fig. 4). More than 80% of fibroblasts were induced to reorient themselves in the two quadrants of ±(45 to 90)° as shown in Fig. 4b. In contrast, rat MSCs were found not significantly reoriented in response to electrical stimulus (Fig. 4c). Application of an even stronger electrical stimulus (e.g., 10 V/cm) failed to induce significant MSC reorientation. Consistent with this observation, the number of rat MSCs found in the 4 quadrants was approximately the same (Fig. 4d).

Induced cell reorientation appears to depend on the cell type, suggesting differential cell adhesion in the 3D collagen scaffold.

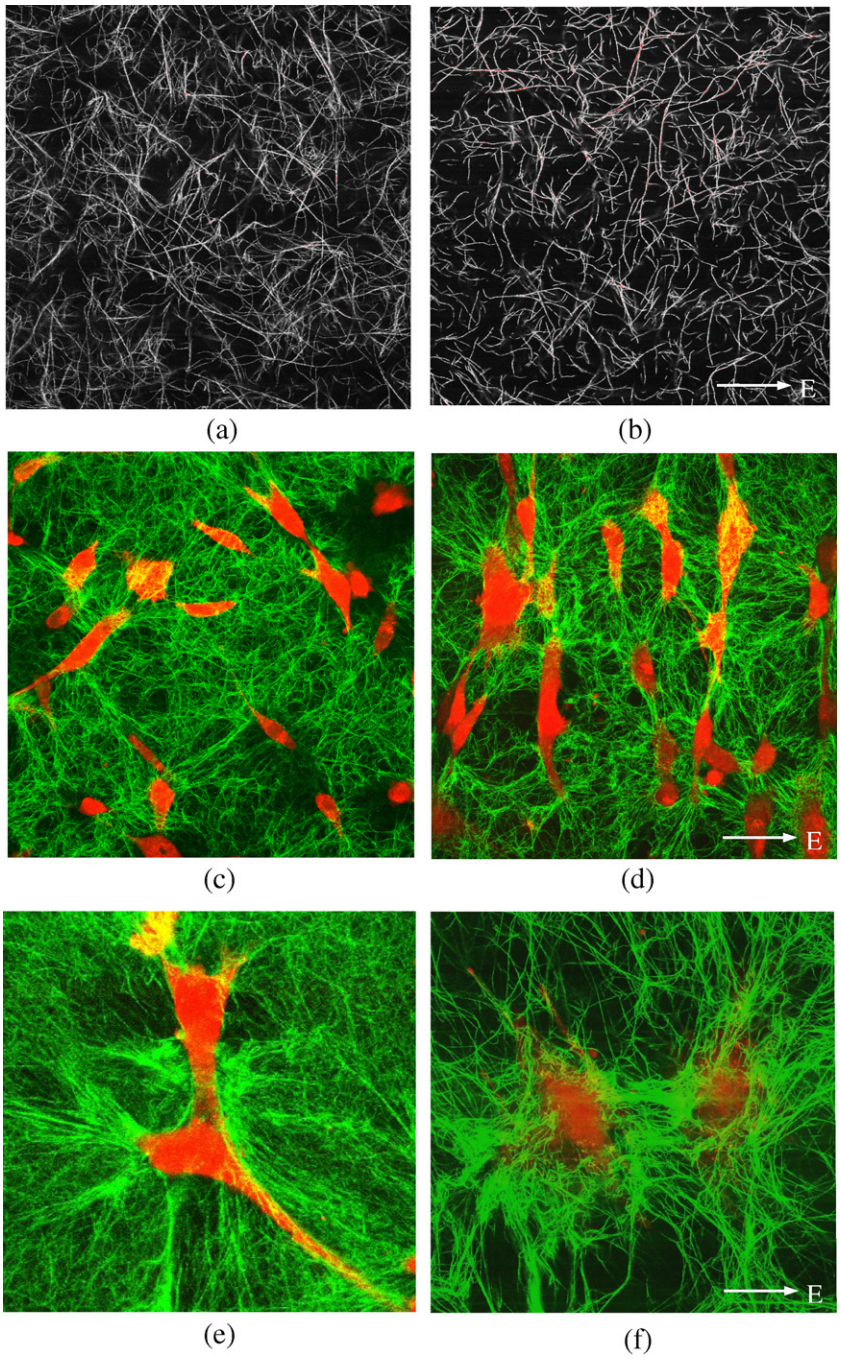

Fig. 5. Images of collagen fibers and cells in response to electrical stimulus. The cells were stained with the fluorescence cell tracker CMTMR. Collagen fibers were visualized using a Bio-Rad femtosecond laser scanning multiphoton microscope. Second harmonic generation (SHG; no fluorophore required) signals from collagen fibers were excited by a wavelength of 840 nm beam. The formation of SHG radiation, which occurs at half of the incident laser wavelength, was monitored with broadband filters (340±70 nm) in front of the detector. (a) and (b) are the cell-free collagen fibers before and after exposure to an electrical stimulus (10 V/cm, strongest stimulus used in our experiments), respectively. (c) and (d) demonstrate the orientation of fibroblasts in the 3D collagen scaffold without and with an exposure to 7 V/cm stimulus, respectively. Finally, (e) and (f) represent images of rat MSCs in the 3D collagen scaffold without and with an exposure to 10 V/cm stimulus, respectively. The arrow indicates the direction of electric field vector.

To determine the interactions between the collagen and cells seeded in the collagen scaffold, images of the cells and fibrous collagen structures were obtained simultaneously using a laser scanning multiphoton microscope. While the collagen structures were imaged using the second harmonic generation (SHG) technique [24,25] that requires no fluorophores, either fibroblasts or rat MSCs were loaded with CellTracker before seeding the cells in the collagen scaffold. First, we tested the possible direct interactions between collagen fibers and electrical stimulus. A collagen gel was prepared as described but no cells were seeded. The cell-free collagen gel was exposed to an electrical stimulus and the collagen fibers were imaged. As shown in Fig. 5, the collagen fibers remained essentially the same before (Fig. 5a) and after (Fig. 5b) the exposure to an electrical stimulus without noticeable fiber reorientation or alignment, indicating that collagen fibers themselves are not likely influenced by electrical stimulus. Second, when fibroblasts were seeded in the 3D collagen scaffold, the cells were found to be spindle-like shape (Fig. 5c), and this effect on cell morphology became more pronounced in response to application of an electrical stimulus (Fig. 5d). While the collagen fibers appear to bind to fibroblasts, the overall collagen orientation was entangled and random before exposure to an electrical stimulus (Fig. 5c). In response to electrical stimulus, not only the collagen fibers were seen to be reorganized but also there appears to be a preferential collagen fiber alignment along the direction of the electrical stimulus (i.e., horizontal axis). Third, rat MSCs seeded in a 3D collagen scaffold show different cell adhesion characteristics. For example, as shown in Fig. 5e, rat MSCs did not show the spindle-like cell shape in the collagen gel, and not random but clearly concentrated collagen fiber bundles are involved in rat MSC adhesion, suggesting tighter connection between MSCs and the surrounding microenvironment. Intense and oriented collagen fiber bundles were particularly noticeable around the cell periphery. In response to the same electrical stimulus that caused fibroblasts to reorient and collagen fibers to preferentially realign, neither reorientation of rat MSCs nor collagen fiber realignment was obvious. Although collagen fiber bundles around the rat MSC periphery seem to have been disorganized and became disturbed, the concentrated collagen fibers remained intact and visible after exposure of the electrical stimulus.

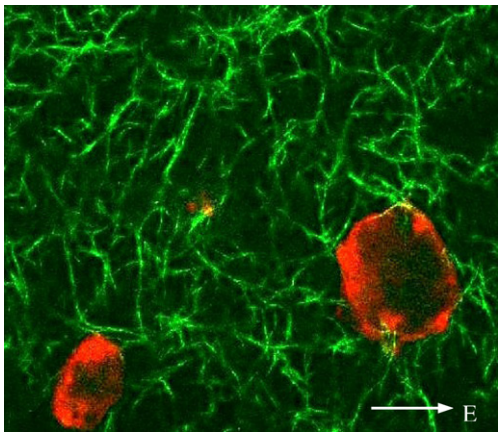

Fig. 6. Role of integrin in rat MSCs response to electrical stimulus. Rat MSCs were treated with fluorescence conjugated anti-CD29 antibodies (1 : 100 dilution) to label and block β_1-integrin subunits prior to the exposure to a 10 V/cm stimulus for 60 min. This antibody treatment caused the cells to become morphologically round and lose physical connections with the collagen fibers. Application of the electrical stimulus induced no further discernable changes in the cell morphology or cell reorientation. The arrow indicates the direction of electric field vector.

Integrin is likely to mediate 3D cell adhesion. To determine the role of integrin in 3D cell adhesion and orientation, rat MSCs were incubated with monoclonal antibodies directed against β_1-integrin (anti-CD29) and then exposed to an electrical stimulus. This antibody treatment caused the rat MSCs to assume round cell morphology, and the bundles of collagen fibers that were shown to mediate strong rat MSC adhesion (Fig. 5e) were not observed (Fig. 6). This result suggests that, similar to integrin-mediated fibroblast adhesion and migration [18], functional integrins are also required to mediate MSC adhesion in the 3D reconstituted collagen scaffold. Indeed, these antibody-treated cells showed lack of specific adhesion sites and may have been spatially confined without molecular attachments.

4. Discussion

Fibroblasts and rat MSCs seeded in the collagen scaffold respond differently to electrical stimulus. While fibroblasts are induced to reorient themselves perpendicularly to the axis of electrical stimulus, rat MSCs exhibit only a limited reorientation. Multiphoton microscopy reveals that collagen fibers are likely responsible for differential cell adhesion. While an entangled network of collagen fibers bound to the fibroblasts is induced to preferentially align with reoriented cells, the physically strong adhesion originally observed between rat MSCs and collagen fiber bundles appear to have been only partially relaxed in response to electrical stimulus without collagen realignment.

At least two physical mechanisms could be postulated to mediate cell orientation in the 3D collagen scaffold. First, alignment of collagen fibers in response to an electrical stimulus could induce cell orientation. Based on analysis of the collagen fiber images revealed by multiphoton microscopy that show no discernable fibril arrays in the bulk of collagen scaffold, this mechanism is unlikely responsible for altered cell shape and reorientation induced by electrical stimulus. Second, conversely, active cells may rearrange localized microenvironment by cell–collagen interactions. Taken together with the findings that cell adhesion and orientation depend on integrins (Fig. 6) and the collagen fiber rearrangement is more pronounced around the cell periphery in response to electrical stimulus, it is likely that localized collagen fiber rearrangement by the cell rather than global scaffold alignment is responsible for cell orientation.

Although electrocoupling mechanisms responsible for changes in cell adhesion and cell reorientation remain to be fully elucidated, several factors are often taken into account. One, the galvanotactic responses are thought to be responsible for such cellular behaviors. Generally, the perpendicular cell reorientation is likely to minimize the perturbing effect of an

electrical stimulus on the membrane potential [13]. Because the electrical potential drop cross the cell is proportional to the cell length along the electric field vector, changes in the membrane potential can be estimated by $\delta V \sim E * L$, where E is the strength of electrical stimulus and L is the cell dimension. In response to a 10 V/cm stimulus, δV across the cell in the direction of an electrical stimulus is estimated ~10 mV (assuming less than 10 μm length in the minor cell axis). Two, since Ca^{2+} flux is known to be involved in the response of cells to the electrical stimulus [26,27], changes in the membrane potential may regulate intracellular Ca^{2+} ion concentration. For example, as a 2 V/cm stimulus is not sufficient to activate voltage-gated Ca^{2+} channels (VGCCs) by inducing ~10 mV change (assume $L = 50$ μm), a stronger stimulus of 10 V/cm can induce δV of 50 mV or greater, which should be sufficient to activate VGCCs and regulate Ca^{2+}-dependent subcellular processes, including cytoskeletal reorganization that is likely to cause changes in the cell morphology and reorientation signaling pathways [28]. Specific molecular mechanisms that are involved in regulation of MSC adhesion and orientation in response to non-invasive electrical stimulus remain to be identified. In addition to growth factors and GTP-exchanger factor that are required in cell orientation [29,30], integrin also appears to be important in this process. Based on the images that show (1) unusually strong adhesion between MSCs and collagen fibers, (2) clustering of integrins and lack of specific adhesion sites in response to treatment of anti-integrin antibodies (Fig. 6), it is plausible that integrin-mediated adhesions found in MSCs differ from those typically observed in terminally differentiated cells.

In order to further elucidate differential adhesion mechanisms, we have recently performed a membrane mechanical study to provide additional evidence that differential adhesion mechanisms may be involved in MSCs. Using laser optical tweezer (LOT) technique [31], the membrane tethers were formed and stretched optically using an infrared laser. As shown in Fig. 7, when the same amount of optical force was applied, the tethers formed in the MSC membrane were much longer than those formed in the fibroblast membrane. Because the formation of these tethers has been shown to depend on the membrane tension and membrane interaction with cytoskeleton [32], these new findings suggest that the MSC membrane may not be tightly coupled to the cytoskeleton. This postulate offers an explanation that, because a close interplay between the membrane and cytoskeletal organization is required to mediate changes in the cellular morphology and cell reorientation [33], loosely connected membrane and cytoskeleton observed in the MSCs but not in the fibroblasts may prevent the electrically induced cellular responses. Together with strong and integrin-mediated adhesion and weak membrane mechanical properties, the usual electrocoupling mechanisms that are sufficient to explain the galvanotatic responses of terminally differentiated cells would have to be modified for MSC adhesion and reorientation. More

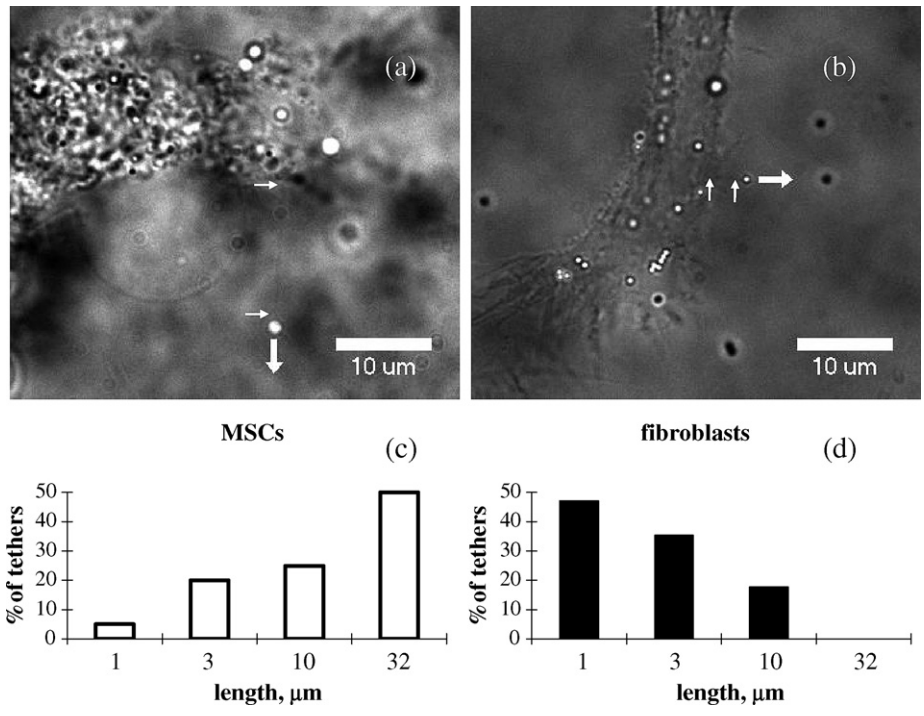

Fig. 7. Plasma membrane tether formation by laser optical tweezers (LOT). Bright-field images of membrane tethers pulled from (a) MSCs and (b) HT1080 fibroblasts, respectively. Polystyrene beads (0.5 μm diameter) conjugated with anti-CD29 were attached to the membrane and pulled away with infrared laser optical trap at a constant speed, as indicated by arrows. The magnitude of optical forces required to form these membrane tether was 3 to 10 pN. Tether length distributions for the two types of cells are shown in (c) and (d). Much longer tethers may be produced from stem cells than from fibroblasts using the same LOT force. The tether length is the distance pulled by LOT where the optical force is exceeded by tether tension and the bead is escaped from the optical trap. Each histogram represents data for 30 tethers from at least 15 cells.

systematic studies are currently underway to examine the role of membrane mechanics that may regulate the cell-type dependent adhesion and reorientation.

In summary, application of an electrical stimulus causes fibroblasts to change cell shape and reorient in the 3D collagen scaffold perpendicularly to the direction of electrical stimulus, accompanied by a preferential realignment of collagen fibers. The same electrical stimulus applied to MSCs induces much less significant reorientation or collagen fiber realignment. Stronger rat MSC-collagen binding has been revealed using multiphoton microscopy, and such tight interactions can be at least partially relaxed using an electrical stimulus. Cell orientation is unlikely induced by fibril arrays in the bulk of collagen scaffold in response to electrical stimuli. Electrocoupling mechanisms that have been found to mediate changes of cell morphology and reorientation of terminally differentiated cells (i.e., fibroblasts) may not be directly applicable to MSCs. This may be attributed to differential cell adhesion mechanisms. However, when treated with integrin antibodies to block the function of integrin, both terminally differentiated fibroblasts and rat MSCs appear to have lost the ability to response electrical stimulus, indicating that integrin-mediated mechanism is likely to regulate 3D cell morphology and orientation. Based on the membrane tether formation study, the mechanical properties of the MSC membrane are not similar to those in the fibroblast and may offer an explanation for atypical MSC responses. Finally, optimal application of electrical stimulus could offer a novel engineering technique to regulate cell type-dependent cellular shape and orientation that are known to be involved in cell differentiation and growth. It also can be of potential use to selectively control a population of cells in co-culture environment.

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