Directional Dependence of Cyclic Stretch-induced Cell Migration in Wound Healing Process of Monolayer Cells

Kazuaki Nagayama,* # Yuya Suzuki,** Daisuke Fujiwara**

Abstract Cells sense the mechanical properties of their surrounding environment and activate intracellular signaling pathways that play important roles in cell survival, proliferation, differentiation, and migration. Migration of cells into an injury site is crucial for repair after injury and requires cytoskeletal reorganization and remodeling of focal adhesions that connect the cytoskeleton to the extracellular matrix. Thus, it is possible that a directional cyclic stretch stimulation of cells may facilitate the wound healing process and establish ordered tissue formation. Here, we investigated the effects of directional cyclic uniaxial stretch on wound repair processes of monolayer epithelial-like cells that was scratch wounded. We controlled the direction of scratched wound in cell tissue to be i) perpendicular to the stretch direction (perpendicular stretch), ii) parallel to the direction of the zero normal strain in the substrate θ₀ (~60°) (oblique stretch), and iii) parallel to stretch direction (parallel stretch). We found that cyclic stretching perpendicular to the scratched wound direction did not improve cell migration, whereas oblique stretching, by which cells were induced to align in the zero normal strain direction θ₀, significantly facilitated cell migration for wound closure even though the migration direction was varied. We further found that cell migration for wound closure was improved most efficiently by cyclic stretching parallel to the wound direction, which facilitated polymerization of actin cytoskeleton aligning in the migration direction and vinculin–actin interactions. These results indicate that cell migration for wound healing is significantly influenced not only by the normal strain applied to cells but also by shear strain under cyclic strain fields, and cells for wound healing preferentially migrate to the direction in which both the normal and shear strains applied to them become smaller.

Keywords: cell biomechanics, mechanobiology, wound healing, actin reorganization, focal adhesions.

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1. Introduction

Cells sense the mechanical properties of their surrounding environment and activate intracellular signaling pathways that play important roles in cell survival, proliferation, differentiation, and migration [1]. Migration of cells into an injury site is a primary component of repair after injury, defining the physical barrier between the host and external environment. Cell migration during wound healing involves coordination and polarization of the leading edge of the cells, cytoskeletal reorganization, and remodeling of focal adhesions, which requires dynamic force-responsive protein complexes such as vinculin, talin, and integrins that couple the extracellular matrix to the cytoskeleton [2]. Interactions between actin cytoskeleton and focal adhesion proteins play essential roles in the mechanical responses of cells and regulation of these cellular functions. Several studies demonstrated the influence of mechanical stimulation on cell migration during wound healing: a sustained unidirectional stretching facilitated wound healing by bovine aortic endothelial cells [3], whereas a biaxial unidirectional cyclic stretching of cells significantly inhibited wound repair of alveolar cell monolayers via actin disruption and reduction of actin-vinculin colocalization [4]. However, biaxial unidirectional stretching produces tensile strains in all directions in the sheet plane, which is quite different from the directional uniaxial stretching. Thus, the mechanical effects of directional cyclic stretch stimulation of cells on wound healing processes are unclear at this stage.

It has been reported that almost all types of cells adhering to an elastic substrate elongate and re-align their
actin cytoskeletons nearly perpendicular to the direction of the stretch when they are subjected to uniaxial cyclic stretching. Actin stress fibers re-orient in the direction of zero normal strain so as to minimize the stress or strain applied to them. These reorientation responses have been investigated in detail by many research groups using various types of cells including endothelial cells [5, 6], smooth muscle cells [7], fibroblasts [8], and osteoblasts [9, 10]. Thus, it is possible that directional cyclic stretch stimulation of cells may facilitate the wound healing process and establish ordered tissue formation.

The purpose of this study was to investigate the effects of directional cyclic uniaxial stretching on wound repair processes of monolayer of epithelial-like cells that were scratch wounded. We controlled the direction of scratched wound in three ways with respect to stretch direction, measured cell migration behavior using our originally designed stretching apparatus mounted on a microscope stage, and analyzed cell migration distance and direction against the scratch direction. We further investigated the changes in expression and distribution of actin cytoskeletons and vinculin focal adhesions in the three groups of scratch-wounded cells subjected to cyclic stretching, in order to assess their wound healing ability.

2. Methods

2.1 Cell culture

*Xenopus laevis* epithelial-like cells derived from tadpoles (XTC-YF, RCB0771; RIKEN BioResource Center, Tsukuba, Japan), which are widely used for studying animal development, regeneration, and wound healing [11, 12], were cultured in a mixture of 50% Leibovitz’s L-15 medium (128-06075; Wako), 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, USA), and 40% distilled deionized water supplemented with penicillin and streptomycin (Sigma, St. Louis, MO, USA) by the following equations,

\[
\varepsilon(\theta) = \frac{\varepsilon_1 + \varepsilon_2}{2} + \frac{\varepsilon_1 - \varepsilon_2}{2} \cos 2\theta \quad (1)
\]

\[
\gamma(\theta) = (\varepsilon_1 - \varepsilon_2) \sin 2\theta \quad (2)
\]

where \(\varepsilon_1\) is tensile strain in the stretch (\(x\)) direction and \(\varepsilon_2\) is compressive strain in the transversal horizontal (\(y\)) direction. Considering directional symmetry, the direction of zero normal strain in our silicone chamber (B). Considering directional symmetry, the direction of zero normal strain of \(\theta_0\) and \(\theta_\pm\) were defined (C). Diagrammatic illustration of the cyclic strain waveforms used in this study (D).

2.2 Cell culture chamber for cyclic stretching

Silicone rubber membranes made with poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow-Corning) were used as elastic substrates for cyclic stretching (Fig. 1A). Using a PDMS liquid as glue, a 0.1-mm-thick silicone rubber sheet (23 × 44 mm) was glued to the bottom of a 10-mm thick rectangular silicone rubber frame (23 mm × 44 mm and 20 mm × 20 mm, outside and inside dimensions, respectively) to form a cell culture chamber. A certain threshold amplitude for reorientation of cells was observed between 2% and 3.5%, below which no reorientation occurred [13]. Thus, in this study, the silicone sheet in the chamber was stretched cyclically by 5%.

This caused deformation of the sheet not only in the stretch (\(x\)) direction but also in the transversal horizontal (\(y\)) direction due to Poisson effects. The strain in the center portion of the sheet (−10 mm square) was measured by tracking benchmarks on the sheet. The actual strain in the sheet was found to be 5.1 ± 0.4% (mean ± SD, n = 8) in the \(x\) direction, and −1.9 ± 0.3% in the \(y\) direction. Using these strain values, the two-dimensional normal strain \(\varepsilon(\theta)\) and shear strain \(\gamma(\theta)\) of the silicone sheet in the direction \(\theta\) under uniaxial stretching is represented by the following equations.
this study, we used trapezoid waveform stretching (Fig. 1D), because this waveform significantly facilitated the reorientation response of cells [10].

2.3 Cell stretching apparatus for real-time imaging
A homemade cell stretching apparatus set on the stage of an inverted microscope (IX71, Olympus, Japan) was used. We developed the apparatus to stretch cell tissue symmetrically using a mechanism with left- and right-handed screws (Fig. 2A, B). These screws were rotated with a stepping motor (PK543-A, Oriental Motor, Japan) controlled by a motor controller (STB-140, Strex, Japan) connected to a personal computer. We were able to observe the same cells continuously in the chamber during cyclic stretching.

2.4 Observation of wound healing processes of cellular tissue in cyclic strain fields

The XTC-YF cells were seeded on the silicone sheets coated with fibronectin (100 mg/mL) at constant density (50 cells/mm²), and precultured statically at 25°C for 2–3 days until the cells reached confluence. The cell monolayers were scratch-wounded using a toothpick-based handmade scratcher, yielding approximately 600-µm width linear scratches. The cells were then subjected to a 5% simple uniaxial cyclic stretch at 1 Hz for 12 h at room temperature (25°C) using the above-mentioned cell stretching apparatus mounted on the stage of the inverted microscope. Phase contrast images of cells were then captured at 15-min intervals using a 10 × objective lens [numerical aperture (NA) = 0.4] and an electron-multiplying CCD camera (C9100–12, Hamamatsu Photonics) connected to a personal computer to analyze cell migration.

To assess the effects of stretch direction on the wound healing processes of cells, we controlled the direction of scratched wound in cell tissue to be: i) perpendicular to the cyclic stretch direction (perpendicular stretch, Fig. 2C), ii) parallel to the direction of the zero normal strain in the silicone sheet θ₀⁺ (oblique stretch, Fig. 2D), and iii) parallel to the cyclic stretch direction (parallel stretch) (Fig. 2E). We analyzed cell migration distances and speeds using the captured images. We also assessed the cell migration direction φ perpendicular to the direction of the scratch (Fig. 2F). In this study, we used Xenopus laevis cells derived from tadpoles which can be cultured in Leibovitz’s medium at room temperature without a CO₂ incubator, thus allowing time course imaging of cells without additional instruments.

2.5 Immuno/fluorescence

After applying cyclic stretching, the cells were fixed with phosphate buffered saline (PBS, Nissui, Japan) containing 3.7% formaldehyde for 5 min, permeabilized with PBS containing 0.5% Triton X-100 (MP Biomedicals) for 5 min, and finally rinsed with PBS containing 1% bovine serum albumin (BSA) to block nonspecific protein binding. We also incubated the fixed cells with this blocking solution for 30 min before treatment with staining reagents. Fluorescent staining of actin filaments in the cells was performed by incubation with Alexa Fluor 546-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) at a concentration of approximately 200 nM for 60 min. For fluorescent visualization of nuclei, intranuclear DNA was stained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) for 30 min. Fluorescent staining of vinculin, the major protein component of focal adhesions, was performed as follows. Samples
were incubated with mouse antibodies against vinculin (1:200 dilution, ab18058, Abcam) for 1 hour at room temperature, followed by incubation with secondary antibody (rabbit anti-mouse Alexa Fluor 488 (1:200 dilution, Invitrogen) for 1 hour at room temperature. All antibodies were diluted in PBS containing 1% BSA. Fluorescence images of actin filaments, nuclei, and vinculin at the leading edge of the scratch wound region of cells were randomly captured as 512 × 512 pixel images using an imaging system consisting of an inverted microscope with a 40 × objective (NA = 0.9) and an electron multiplying CCD camera. Optical components of the microscope, such as filters and iris diaphragms, were kept under the same conditions during the measurements.

2.6 Statistical analysis
Data are expressed as means ± SD. Data were analyzed using ANOVA with correction for multiple comparisons, followed by Steel-Dwass test for multiple comparisons of the means between two groups, using the web-based free statistical analysis program MEPHAS developed at Osaka university (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/; in Japanese). P values < 0.05 were considered significant for all analyses.

3. Results and Discussion
To examine how the cyclic stretch direction affected cell migration during wound repair, confluent cultured XTC-YF cells were exposed to cyclic stretching following wounding, and phase-contrast images were collected from 0 to 12 h (Fig. 3). After wounding, cell monolayers showed an organized wound front at the leading edge. The wounds were almost closed by 12 h, while the migration rate appeared different among group.

The migration trajectories of the cells were measured using the captured images (Fig. 4), revealing that the migration direction at the leading edge differed considerably among groups. Cells exposed to perpendicular or oblique stretching migrated in a direction oblique to the direction of wound closure (Fig. 4A, B). Especially in oblique-stretched cells, the migration direction exhibited markedly wide variation (Fig. 4B, Table 1). In contrast, the parallel-stretched cells showed relatively linear migration in the direction of wound closure, with small variation (Fig. 4C and Table 1), indicating that these cells achieved the most efficient migration for wound closure. The total migration distances of the cells are shown in Fig. 5. The cells at the wound edge in static conditions migrated approximately 100 µm by 6 h, and the distance was doubled at 12 h. The migration distances of the perpendicular-stretched cells were comparable with those in the static group, while those of the oblique-stretched cells were approximately 270 µm by 12 h, and were significantly greater than the static cells. Furthermore, cell migration was improved most efficiently in the parallel-stretched group, and significant differences compared to other groups were already observed by 6 h. These results indicated that cyclic stretching parallel to the scratch direction remarkably increased cell migration for wound repair.

Previous studies have reported that almost all types of cells adhering to an elastic substrate elongate and re-align their actin cytoskeletons in a direction of zero normal strain, so as to minimize the stress or strain applied to them [5, 8, 10]. Indeed, we confirmed that actin cytoskeletal structures in XTC-YF cells exposed to uniaxial cyclic stretching significantly realigned in the direction of zero normal strain θ0 on the silicone sheet (compare Fig. 6A and B). This reorientation response of actin cytoskeleton is believed to be achieved by minimizing internal normal stress and strain of cells. The cells at the wound edge exposed to perpendicular stretching showed reduced migration for wound closure to the level of the static condition (Fig. 5), because the wound closure direction coincided with the cyclic stretch direction, resulting in disruption of actin cytoskeleton caused by mechanical stretch perturbation. Previous study has demonstrated that unidirectional biaxial cyclic stretching, in which cyclic tensile strain is produced in all directions, significantly inhibited wound repair by alveolar cells via actin disruption and reduction of actin-vinculin colocalization [4]. In this study, perpendicular-stretched
cells showed similar results: the cells at the wound edge exposed to perpendicular stretching exhibited blurred, thin fiber networks of actin cytoskeleton (Fig. 7A), and vinculin focal adhesions did not align in the direction of wound closure (Fig. 7B). In contrast, in the cells exposed to oblique stretching, the wound closure direction coincided with the zero normal strain direction $\theta_{0+}$, which may facilitate migration for wound closure, while the wound closure direction was quite different to the other zero normal strain direction $\theta_{0-}$. Fluorescent images of the oblique-stretched cells revealed that actin cytoskele-

| Cell migration direction with respect to wound closure direction(deg.) | Perpendicular ($n = 80$ cells) | Oblique ($n = 80$ cells) | Parallel ($n = 80$ cells) |
|-------------------------|-----------------------------|------------------------|--------------------------|
|                         | 59.8 ± 38.7                | 53.1 ± 62.4            | 10.2 ± 9.6               |

Fig. 4 Examples of the migration trajectory of cells exposed to cyclic stretching during wound healing processes. The dotted regression lines represent cell migration direction shown in Table 1.

Fig. 5 Migration distance of the cells exposed to cyclic stretching during wound healing processes. Inset images represent the relation between the cyclic stretch direction (double-headed arrows) and scratch wound direction. N and n denote the numbers of measured cell culture chambers and cells, respectively.

Fig. 6 Typical fluorescent images of actin cytoskeletons (red) and nucleus (cyan) in XTC-YF cells in static condition (A) and following exposure to uniaxial cyclic stretching for 12 h (B).
ton aligned in $\theta_0+$ corresponding to the direction of wound closure in some cells (Fig. 7C, white arrowhead), but aligned in $\theta_0-$ in other cells (Fig. 7C, white arrow), and vinculin focal adhesions appear mature and elongated (Fig. 7D, white arrow). The cells aligned in $\theta_0-$ might represent a ‘barrier’ that retards the migration of cells for wound closure, which may account for the wide variation in migration direction in oblique-stretched cells (Fig. 4B, Table 1).

The parallel-stretched cells displayed the most efficient migration for wound closure in this study (Fig. 4, 5, and Table 1). The wound closure direction was perpendicular to the cyclic stretch direction. Thus, the cells migrating for wound closure were not exposed to cyclic stretching, but to cyclic compression, which could disrupt their actin cytoskeletons. However, the compressive strain was only approximately 2% in our case (Fig. 1B, $\theta = 90^\circ$), which was lower than the threshold amplitude for stretch-induced cell reorientation [13], and did not inhibit polymerization and reorientation of actin filaments aligning in the direction of wound closure (Fig. 7E, white arrowheads). Furthermore, in the parallel-stretched group, cells for wound closure migrated perpendicular to the stretch direction. This means that they were not exposed to ‘shear’ deformation during cyclic stretching. This low shear strain is also important to maintain cell-cell adhesion during cell migration for wound closure. The turnover of vinculin is also important to improve cell migration [14]. Vinculin focal adhesions in the parallel-stretched cells were clearly observed at cell peripheries, and were also highly expressed around the nucleus (Fig. 7F). These results indicated that adhesion protein turnover might be higher in the parallel-stretched cells than in the other groups, facilitating cell migration for wound closure. Taken together, cell migration for wound healing is significantly influenced not only by the normal strain applied to cells but also by shear strain under cyclic strain fields, and cells for wound healing preferentially migrate to the direction in which both normal and shear strains applied to them become smaller.

4. Conclusion

We investigated the effects of cyclic stretch direction on wound repair processes of XTC-YF cell monolayer. We found that cyclic stretching perpendicular to the scratched wound direction did not improve cell migration, while oblique stretching, in which cells were induced to align in the zero normal strain direction $\theta_0$, significantly facilitated cell migration for wound closure even though their migration direction showed variation. We further found that cyclic stretching parallel to the wound direction improved cell migration for wound closure most efficiently, with polymerization of actin cytoskeleton aligning in the migration direction and vinculin–actin interactions, under a condition of lower tensile and shear strains during cyclic stretching.

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Kazuaki Nagayama, et al: Directional Dependence of Cyclic Stretch-induced Cell Migration

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