Alignment of Myoblast Cultured on Micro Striped Ridge After Centrifuge Stimulation: Before and After Division

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ABSTRACT

The effect of hysteresis of the tangential force field at the surface of the scaffold on the alignment of myoblast has been studied *in vitro*. The striped pattern (0.7 μm height, 3 μm width, and 3 μm interval) were made on the surface of the scaffold plate to control the orientation of each cell. Variation was made on the angle between the longitudinal direction of the ridge and the direction of the tangential force: 0 degree, 45 degrees, and 90 degrees. C2C12 (mouse myoblast cell line) was used in the experiment. To apply the tangential force field (< 100 G) to the cells, the scaffold plate was set in the tube in a conventional centrifugal machine placed in an incubator. After the centrifugation for 5 hours, the behavior of each cell at the time-lapse microscope images was traced for 24 hours to analyze the angle between the longitudinal axis of the cell and the direction of the past centrifugation. The experimental results show that the tendency of cells to align the longitudinal direction of the striped pattern is strengthened by the hysteresis of the exposure to the tangential force field. The tendency is strengthened at the division of each cell.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravity, Orientation and C2C12.

1. INTRODUCTION

The recent cell culture technique enables observation of the behavior of each cell *in vitro*. A biological cell adheres on the scaffold and shows several active behaviors: migration, deformation, and proliferation. The cell is deformed by the force, because of its compliance. The cell deforms, on the other hand, to minimize the intra force of itself. The cell is moved by the force. The cell, on the other hand, moves by itself. C2C12 is cell line of mouse myoblast. In the previous study, both the migration and the deformation of C2C12 were restricted by the wall shear stress higher than 3 Pa in flow *in vitro* [1]. Deformability of C2C12 was observed by the slit in the previous study *in vitro* [2, 3]. C2C12 extended along the lines of micro ridges on the scaffold surface [4]. Vibration decelerated adhesion of C2C12 on the micro ridges [5]. C2C12 migrated regardless of the direction of the vibrating micro ridges. The orientation of C2C12 was tried to be measured by the electric impedance *in vitro* [6]. Both differentiation and growth of C2C12 were delayed with electric pulses in the previous study *in vitro* [7]. The floating cells of C2C12 were tried to be sorted by the micro groove on the wall of the flow channel [8].

These behaviors might depend on the history of each cell. While the strong stimulation above the threshold damages the cell, the stimulation below the threshold remains in the cell as a memory for the response in the next step. The memory might be reset after division of the cell. To trace the hysteresis effect of the mechanical stimulation on the single cell, the time-lapse images are effective. In the previous study, the longitudinal axis of C2C12 (mouse myoblast cell line) tends to align to the direction of the excess gravity. The axis tilts to the perpendicular direction, on the other hand, after stopping of the excess gravity [9]. A single cell migrates at random on the scaffold. The cell tends to align to the longitudinal direction of the micro ridge line [10].

In the present study, centrifugal force has been used to apply the tangential force field on the surface of the scaffold *in vitro*. The hysteresis effect of the force field on the single cell attached on the scaffold has been investigated, after stopping of the stimulation of the excess gravity. To examine the effect of the direction of the force field on the cell at the specific alignment, the orientation of the cell was controlled by the striped pattern on the surface of the scaffold. The behavior of each cell has been traced before and after division.

2. METHODS

Micro-pattern on Scaffold Plate

The micro striped pattern (Fig. 1) has been made in three partial rectangular areas of 0.4 mm × 1.6 mm on the PDMS (polydimethylsiloxane) plate of the scaffold by photolithography technique. The height (H), the width (W), and the interval (I) of the quadrangular ridges are 0.7 μm, 3 μm, and 3 μm, respectively. Each area has its own specific direction of the striped pattern. Namely, variation has been made on the angle (α) between the longitudinal direction of the ridge and the direction of centrifuge: 0 degree (parallel), 45 degree, and 90 degree (perpendicular). Three partial area was made on the same surface of the scaffold plate in parallel position, so that the behavior of cells on each area can be

compared simultaneously (Fig. 2). The pattern of each area was also used as a marker to trace each cell.

Mold for Micro Pattern

The borosilicate glass (Tempax) disk was used for the base of the mold through micromachining process. The positive photoresist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the glass with the spin coater (at 3000 rpm for 20 s). The micro pattern was drawn on the mold with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for two minutes. The glass plate with the photoresist material was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan).

After the mold of the glass disk was enclosed with a peripheral wall of polyimide tape, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was poured with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA) on the mold. The volume ratio of PDMS to curing agent is ten to one. The baked plate of PDMS (1 mm thickness) was exfoliated from the mold of the slide glass plate. The PDMS plate was cut to make a plate of 15 mm × 10 mm × 1 mm, and stacked on the glass plate of 50 mm × 13 mm × 1 mm. The surface of the PDMS plate was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) in the reactive ion etching system (FA-1: oxygen plasma ashing, 50 W) for thirty seconds just before the cell culture.

Tangential Force Field

The tangential force field was applied to the culture surface with the centrifugal force. The culture plate is inserted into the tube, which is contained in the rotor. The angle between the radial direction of the rotation of the rotor and the axial direction of the tube in the rotor is 60 degree. The glass plate was set so that the culture surface is parallel to the radial direction of the rotation. The centrifugal force is applied parallel to the culture surface. The centrifugal force (F_c) is calculated by Eq. (1).

$$F_c = m\, r\, \omega^2 \tag{1}$$

In Eq. (1), m is mass, r is radius of the rotation, and ω is angular velocity. In the gravitational field, gravitational force (F_g) is calculated by Eq. (2), where g is gravitational acceleration.

$$F_g = m\, g \tag{2}$$

In the present study, the centrifugal acceleration lower than 100 G (1 G is equal to the gravitational acceleration) ((Fc / Fg) = 100) is applied with the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan).

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the test. C2C12 of the passage four was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. The cells were seeded on the glass plate at the density of 3000 cells/cm^2. After the cells were cultured for 12 hours in the resting state, excess gravitational stimulation was applied for five hours. The gravity of 50 G (r = 0.045 m, group X), and the gravity of 100 G (r = 0.09 m, group Y) were applied at ω = 104 rad/s. To apply the excess gravity, the plate, on which cells were cultured, was set in the medium in the tube. Several tubes with the glass plate were set in the rotor of the centrifugation to cultivate cells, simultaneously. To keep the content of carbon dioxide of 5 % at 310 K, the cells were cultured in an incubator through the entire experimental term including the term of exposure to the hyper-gravitational field. Namely, the centrifugal machine was placed in the incubator during the centrifugation.

In the control group, the cells were cultured without centrifuge on the plate, which was placed in the polystyrene dish. In the control group, no tangential force field was applied, and only the gravitational force (1 G) normal to the scaffold surface plate was applied.

Image Analysis

After stimulation of the excess gravity, cells on the glass plate were moved from the centrifugal tube to the culture dish. Cells were subsequently cultured in the dish placed in the incubator combined with an inverted phase-contrast microscope (LCV110-SK, Olympus Co., Ltd., Tokyo). The behavior of each cell was analyzed on the time-lapse images captured every ten minutes for 24 hours after stimulation of the excess gravity. Cells, which made division, were traced: before and after division. "Image J" was applied to analyze the behavior of each cell. On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid (Fig. 1). On the ellipsoid, the length of the major axis (a), and the minor axis (b) were measured. The ratio of axes is calculated as the shape index (P) by Eq. (3).

$$P = 1 - b / a \tag{3}$$

At the circle ($a = b$), $P = 0$. As the ellipsoid is elongated ($a >> b$), P approaches to 1. The acute angle ($0 < \theta < 90$ degree) between the direction of the centrifuge and the direction of the major axis of each cell was measured at the microscopic image. When the major axis is parallel to the direction of centrifuge, $\theta = 0$. When the major axis is perpendicular to the direction of centrifuge, $\theta = 90$ degree.

3. RESULTS

By the time-lapse images, continuous activity of each cell was confirmed: migration, deformation, and division (Fig. 2). Each cell repetitively extends pseudopods. The centrifuge direction was easily found by the stripe patterns of 0 degree. The behavior of each cell was easily traced with the help of the stripe patterns as markers. Each cell shows active behavior on micro striped ridges: migration, deformation, and division.

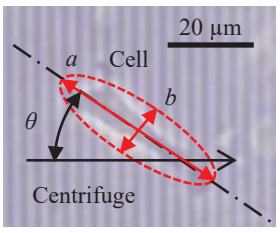

Fig. 1: On microscopic image, outline of each cell was traced, and contour of each cell was approximated to ellipsoid: on stripe ridge lines (α = 90 degree).

Figs. 3–5 show tracings of the angle (θ) of each cell. Each cell divides at the time zero. Each tracing of two tracings after division show tracing of each divided cell. Fig. 3 shows the angle of cell without centrifugal stimulation. Without the tangential force stimulation, the angle (θ) of each cell tends to align to the direction of striped ridges (Fig. 3). The tendency to align parallel to the tangential force field does not affect the alignment of each cell one hour after stimulation (Figs. 4 & 5). Each cell tends to align to the direction of the striped ridges, except just after division. On the striped ridges parallel to the tangential centrifugal force field of 100 G, each cell remarkably aligns to the direction of the striped ridges after the division (Fig. 5a). Cells often make opposite alignment each other just after the division (Figs. 3b & 4b).

Figs. 6–8 show tracings of the shape index (P) of each cell. The shape index (P) decreases immediately after division, which corresponds to approaching to the spherical shape. On the striped ridges parallel to the tangential centrifugal force field of 100 G, on the other hand, the shape index increases occasionally (Fig. 8a).

Data are rearranged as the relationships between the shape index (P) and the angle (θ) of each cell after 100 G in Fig. 9. The regression line shows the tendency that the shape index (P) decreases with the angle (θ) on the striped ridges of 0 degree (α) (Fig. 9a). The tendency corresponds to the elongation of the cell to the longitudinal direction of the ridge. The correlation coefficient (r^2) is higher before the cell division. The tendency is not clear after the cell division: small value of the correlation coefficient. The decrease of the shape index (P) with the angle (θ) is not clear in Fig. 9b. That corresponds to the unclear tendency of the elongation of the cell to the longitudinal direction of the ridge (α = 90 degrees).

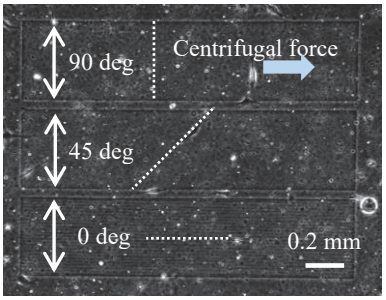

Fig. 2: Cells after stopping centrifuge: α = 90 degree (upper), 45 degree (middle), and 0 degree (lower): dotted line shows direction of ridges.

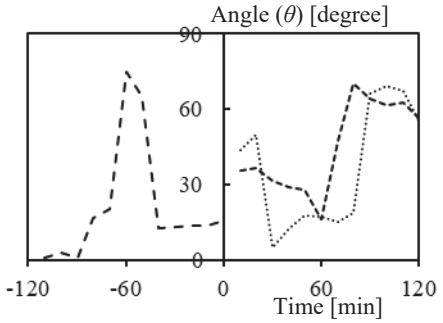

Fig. 3a: Angle (θ) of C2C12: control, α = 0 degree.

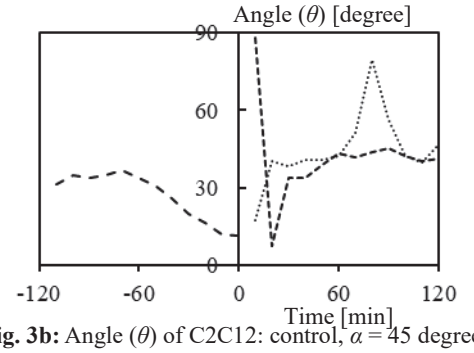

Fig. 3b: Angle (θ) of C2C12: control, α = 45 degree.

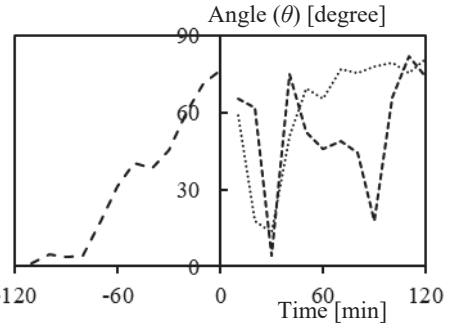

Fig. 3c: Angle (θ) of C2C12: control, α = 90 degree.

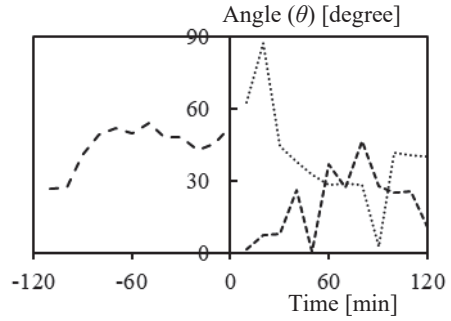

Fig. 4a: Angle (θ) of C2C12: 50 G, α = 0 degree.

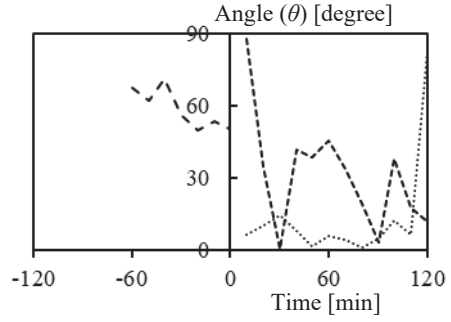

Fig. 4b: Angle (θ) of C2C12: 50 G, α = 45 degree.

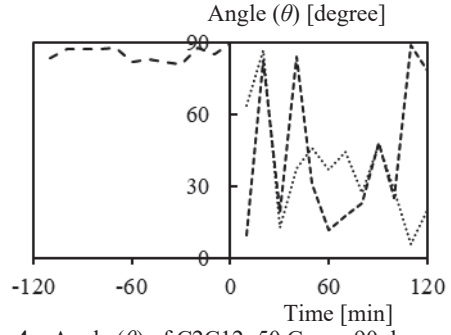

Fig. 4c: Angle (θ) of C2C12: 50 G, α = 90 degree.

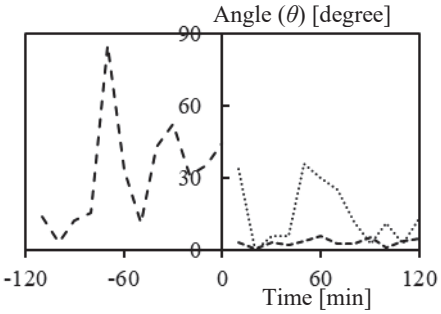

Fig. 5a: Angle (θ) of C2C12: 100 G, α = 0 degree.

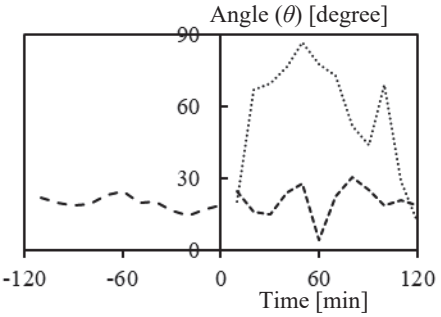

Fig. 5b: Angle (θ) of C2C12: 100 G, α = 45 degree.

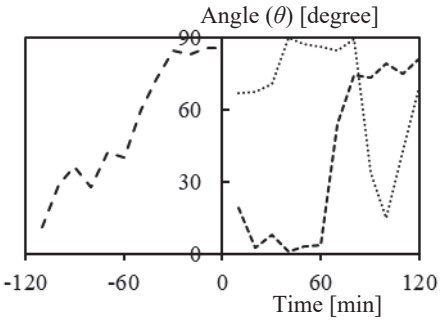

Fig. 5c: Angle (θ) of C2C12: 100 G, α = 90 degree.

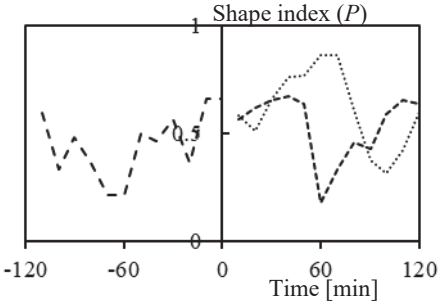

Fig. 6a: Shape index (P) of C2C12: control, α = 0 degree.

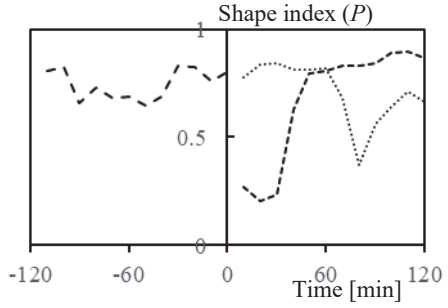

Fig. 6b: Shape index (P) of C2C12: control, α = 45 degree.

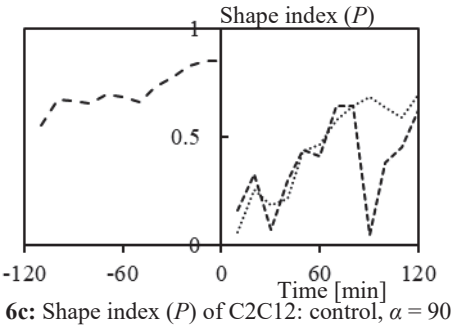

Fig. 6c: Shape index (P) of C2C12: control, α = 90 degree.

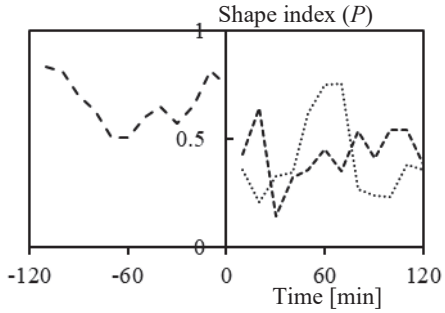

Fig. 7a: Shape index (P) of C2C12: 50 G, α = 0 degree.

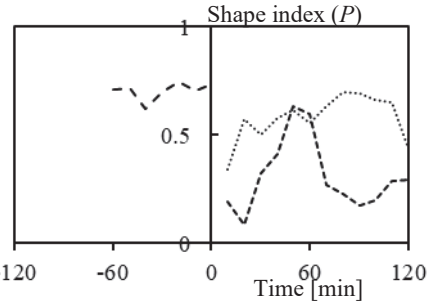

Fig. 7b: Shape index (P) of C2C12: 50 G, α = 45 degree.

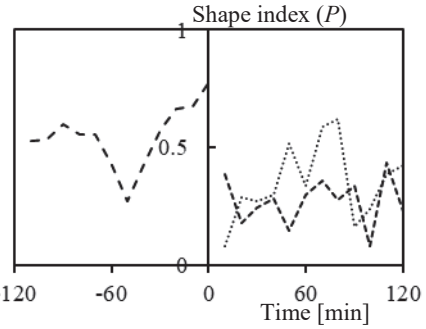

Fig. 7c: Shape index (P) of C2C12: 50 G, α = 90 degree.

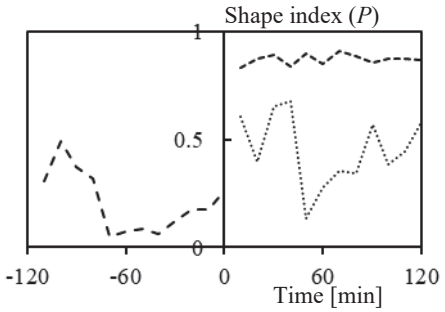

Fig. 8a: Shape index (P) of C2C12: 100 G, α = 0 degree.

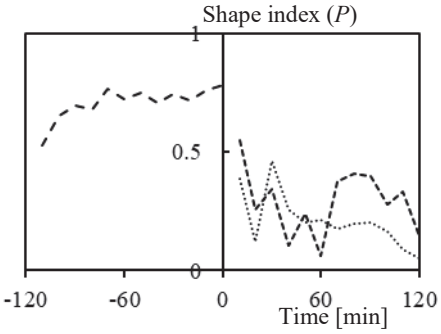

Fig. 8b: Shape index (*P*) of C2C12: 100 G, α = 45 degree.

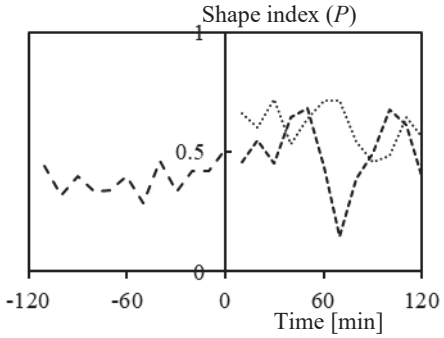

Fig. 8c: Shape index (*P*) of C2C12: 100 G, α = 90 degree.

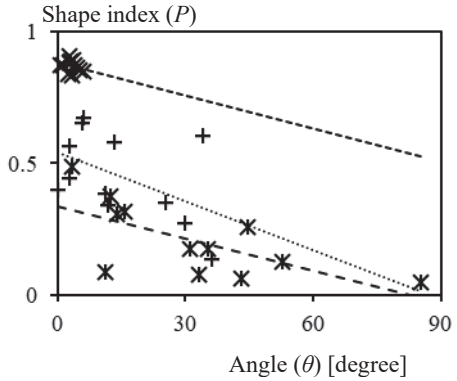

Fig. 9a: Shape index (*P*) vs. angle (θ) of C2C12: 100 G, α = 0 degree (Fig. 5a, 8a): before (asterisk, r^2 = 0.45), after (plus, r^2 = 0.23; cross, r^2 = 0.08) division.

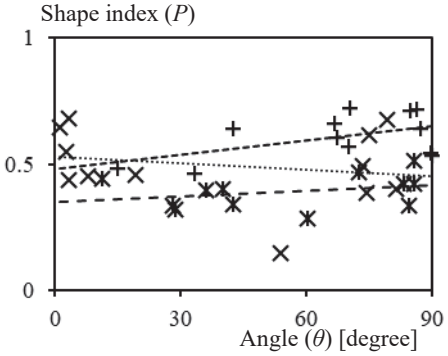

Fig. 9b: Shape index (*P*) vs. angle (θ) of C2C12: 100 G, α = 90 degree (Fig. 5c, 8c): before (asterisk, r^2 = 0.09), after (plus, r^2 = 0.25; square, r^2 = 0.04) division.

4. DISCUSSION

The hysteresis of centrifugation might make each cell increase the area contact with the surface of the scaffold. Each cell might adapt to the tangential force field by maximization of the contact area between the cell and the scaffold.

To control the alignment of the single cell on the scaffold before stimulation, the micro stripe pattern has been used in the present study. The height of the ridge is designed as 0.7 μm, which is slightly higher than threshold to make orientation of cells [10]. The average diameter of the floating cell in the medium is 20 μm. The low ridge and the narrow width were selected so as not to disturb cell migration to every direction. Both deformation and migration of C2C12 are at random over the striped pattern in the present study.

C2C12, on the other hand, tended to elongate and align along the striped pattern even in the case without the tangential force stimulation in the previous study [9]. The tilting tendency of C2C12 was enhanced by the tangential force of 100 G. C2C12 tends to be elongated during the tangential force stimulation. The mild tendency was observed on MC3T3-E1 in the previous study [11].

The behavior of each cell can be easily traced with the aid of the micro striped pattern on the scaffold as the marker. The tracking ability both to the tangential force and to surface morphology was higher in C2C12 than in MC3T3-E1 in the previous study. The tracking ability of 3T3-L1 is much lower than that of MC3T3-E1 [11]. On the striped pattern parallel to the tangential force (0 degree), every type of cell is elongated along the striped pattern. The tracking ability on another direction of striped pattern depends on the cell types.

In the tangential force field, each cell tends to make stronger contact to the scaffold, which makes orientation of cells along the micro striped patterns parallel to the tangential force. In the previous study, C2C12 was most frequently elongated on the striped pattern of 45 degree between the tangential force direction and the longitudinal direction of the stripe pattern [9].

C2C12 differentiation might be accelerated by the mechanical stimulation in the tangential force field by the centrifugal field. The myotube might align to the direction perpendicular to the centrifugal field. Hypergravity affects myoblast [11, 12]. Hypergravity affects tissue formation [13, 14]. The effect was studied with design of scaffold [15]. The effect of hypergravity depends on cell types [16]. The effect of mechanical stress affects molecular response [17–20]. The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro*. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology.

5. CONCLUSION

The effect of hysteresis of the tangential force field at the surface of the scaffold on the alignment of myoblast has been studied *in vitro*. The striped pattern (0.7 μm height, 3 μm width, and 3 μm interval) were made on the surface of the scaffold plate to control the orientation of each cell. To apply the tangential force field (< 100 G) to C2C12 (mouse myoblast

cell line), the scaffold plate was set in a conventional centrifugal machine placed in an incubator. The experimental results show that the tendency of cells to align the longitudinal direction of the striped pattern is strengthened by the hysteresis of the exposure to the parallel tangential force field. The tendency is reset after the rounded shape at the division of cell.

ACKNOWLEDGMENT

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