Alignment and Deformation of MC3T3-E1 Cultured on Micro Striped Pattern after Stimulation of Tangential Force Field

Yuji ENDO, Shigehiro HASHIMOTO, Takuya TAMURA

Biomedical Engineering, Department of Mechanical Engineering,
Kogakuin University, Tokyo, 163-8677, Japan
http://www.mech.kogakuin.ac.jp/labs/bio/

ABSTRACT

The effect of hysteresis of the tangential force field on the single cell cultured at the surface of the scaffold with the micro striped pattern has been studied *in vitro*. The lines of parallel micro quadrangular ridges (0.7 μm height, 3 μm width, and 3 μm interval) were made on the surface of the scaffold plate by the photolithography technique to control the orientation of each cell. Variation was made about the angle between the longitudinal direction of the ridge and the direction of the centrifugal force field: 0 degree, 45 degrees, and 90 degrees. To apply the tangential force field (50 G, 100 G) on the surface of the scaffold, the plate was set in the tube in a conventional centrifugal machine placed in an incubator. After the centrifugation for 5 hours, the contour of each cell at the time lapse microscope images was traced for 24 hours in the incubator, and the angle between the longitudinal axis of the cell and the direction of the centrifugation was analyzed. The experimental results show that the MC3T3-E1 (mouse osteoblast precursor cell) elongates to the longitudinal direction of the striped pattern by the hysteresis of the exposure to the tangential force field more frequently at 100 G than at 50 G.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravity and MC3T3-E1.

1. INTRODUCTION

The microgravity environment might weaken the mechanical supporting system of the biological body. MC3T3-E1 is the cell line of mouse osteoblast precursor cell. The bone remodeling is controlled by the balance between osteoblast and osteoclast. In the previous study, myotube thickness increased in the hyper gravity environment *in vitro* [1]. The shear flow affected migration of osteoblasts in the previous experiment *in vitro* [2]. MC3T3-E1 made orientation along the stream line after the stimulation of the wall shear stress [3]. The floating cells of MC3T3-E1 were tried to be sorted by the micro hole [4] or by the oblique micro groove [5]. 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. 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. To trace the hysteresis effect of the mechanical stimulation on the single cell, the time laps 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, and to turn to the perpendicular direction after stopping of the excess gravity [6, 7]. A single cell migrates at random on the scaffold. The cell tends to align to the longitudinal direction of the micro ridge line [8].

In the present study, the centrifugal force has been used to apply the tangential force field on the surface of the scaffold. The hysteresis effect of the force field on the single osteoblast cell attached on the scaffold has been investigated. To examine the effect of the direction of the force field on the cell at the specific alignment, the orientation of the cell has been controlled by the striped pattern on the surface of the scaffold.

2. METHODS

Micro-pattern on Scaffold Plate

The micro striped pattern 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 (θ) between the longitudinal direction of the ridge and the direction of centrifugal force in the two-dimensional scaffold surface: 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. 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 diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. To remove micro particles on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan). To improve affinity to photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the glass plate at 3000 rpm for 30 s with a spin coater. 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 photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 373 K for ninety seconds.

The pattern for the slit was drawn on the mold with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). The pattern was baked in the oven at 373 K for five minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for two minutes, rinsed with the ultrapure water for three minutes, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

The glass plate with the photoresist material was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of CF_4 (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa. To remove the residual OFPR-800LB on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1).

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. After degassing, PDMS was baked at 338 K for one hour in an oven (DX401). 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 cell culture.

Tangential Force Field

The tangential force field was applied to culture surface with the centrifugal force. The culture plate is inserted in 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, the 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 force is generated with the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan). Variation has been made at the centrifugal force ratio (F_r) by adjusting the radius at the position of the culture plate: 50 G, and 100 G. The value of 1 G means that the centrifugal force is equal to the gravitational force.

$$F_r = F_c / F_g \tag{3}$$

Cell Culture

MC3T3-E1 (passage nine, osteoblast precursor cell line derived from mouse calvaria) was used in the tests. Cells were 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 static state (1 G: normal), the centrifugal force field stimulation was applied for five hours. The centrifugal force field of 50 G (r = 0.045 m) or 100 G (r = 0.09 m) were applied at ω of 104 rad/s.

To apply the centrifugal force field, the plate, on which cells adhered, was set in the medium in the tube. Several tubes (50 G, 100 G) with the glass plate were set in the rotor to cultivate cells, simultaneously [6]. 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 (0 G). Namely, only the gravitational force (1 G) normal to the scaffold surface plate was applied.

After the stimulation of the centrifugation, the glass plate was moved from the centrifugal tube to the culture dish. After the stimulation, no tangential force field was applied (0 G). Only the gravitational force (1 G) normal to the scaffold surface plate was applied. 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 image captured every five minutes for 24 hours after stimulation of the excess gravity.

Image Analysis

"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. 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. 4.

$$P = 1 - b / a \tag{4}$$

At the circle, P = 0. As the ellipsoid becomes flat ($b < a$), P approaches to unity. The angle (0 degree < θ < 90 degrees) between the direction of the centrifugal force 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 centrifugal force, θ = 0. When the major axis is perpendicular to the direction of centrifugal force, θ = 90 degree.

3. RESULTS

By the time laps images, continuous activity of each cell was easily traced: migration, deformation, and proliferation. Each cell repetitively extends pseudopods (Fig. 1). In Figs. 2 & 5, each datum point shows the value of each cell at 12 hours, 17 hours, 23 hours, 29 hours, 35 hours, and 41 hours after seeding.

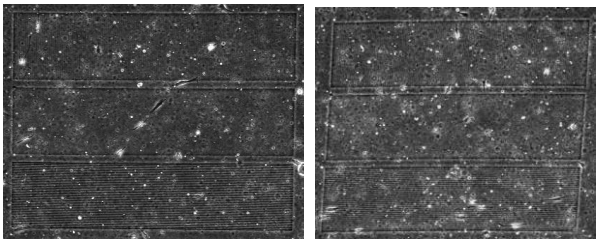

Fig. 1: Cells on striped pattern after 50 G (left) and 100 G (right): 0 degree (upper), 45 degree (middle), and 90 degree (lower): centrifugal force from left to right.

The timing of every 6 hours coincides to the centrifugal force stimulation group. In Figs. 3, 4, 6, & 7, each datum point shows the value of each cell before centrifuge (before), immediately after stopping centrifuge (after), and every 6 hours after stopping centrifuge, respectively: before centrifuge stimulation, immediately after centrifuge stimulation for 5 hours, 6 hours after centrifuge stimulation, 12 hours after centrifuge stimulation, 18 hours after centrifuge stimulation, and after 24 hours after centrifuge stimulation. Fig. 2 shows the direction of the major axis of each cell without centrifuge (control study). Each cell elongates at random orientation on the flat surface without the striped pattern: the angles θ scatters.

Figs 3 & 4 show the angle between the direction of the major axis of each cell and the tangential force (on the surface of the scaffold by centrifuge) of 50 G, and at 100 G, respectively. Fig. 3 shows the angle between the direction of centrifugal force of 50 G and the direction of the major axis of each cell on each micro pattern: 0 degree (Fig. 3b), 45 degrees (Fig. 3c), and 90 degrees (Fig. 3d), respectively. Fig. 3a shows data on the flat surface. The angle tends to increase after stimulation hysteresis. Cells tend to tilt to the perpendicular direction to the centrifugal force after proliferation: after 18 hours. The number of cells decreases around the centrifuge direction. Fig. 3b shows data on the striped pattern parallel to the centrifugal force direction (0 degree). Cells tend to align along the longitudinal direction of the striped pattern, which is parallel to the centrifugal force direction. The tendency is relieved in 12 hours after the centrifugal force stimulation. Fig. 3c shows data on the striped pattern of 45 degree. The direction of each cell scatters around 45 degrees. At 6 hours after the centrifugal force stimulation, each cell tends to tilt to the direction of the centrifugal force. In 12 hours after the centrifugal force stimulation, the direction of each cell scatters again. Fig. 3d shows data on the striped pattern perpendicular to the centrifugal force direction (90 degree). Cells tend to align along the longitudinal direction of the striped pattern, which is perpendicular to the centrifugal force direction. The tendency continues for 24 hours after the centrifugal force stimulation.

Fig. 4 shows the angle between the direction of centrifuge of 100 G and the direction of the major axis of each cell on each micro pattern: 0 degree (Fig. 4b), 45 degrees (Fig. 4c), and 90 degrees (Fig. 4d), respectively. Fig. 4a shows data on the flat surface. The angle tends to increase after stimulation hysteresis. Cells tend to tilt to the perpendicular direction to the centrifugal force after proliferation: after 18 hours. The number of cells decreases around the centrifugal force direction. Fig. 4b shows data on the striped pattern parallel to the centrifugal force direction (0 degree). Cells tend to align along the longitudinal direction of the striped pattern, which is parallel to the centrifugal force direction. The tendency is relieved in 12 hours after the centrifugal force stimulation. Fig. 4c shows data on the striped pattern of 45 degree. The tendency of cells tilting to the direction of striped pattern of 45 degrees at 100 G (Fig. 4c) is higher than that at 50 G (Fig. 3c). Fig. 4d shows data on the striped pattern perpendicular to the centrifugal force direction (90 degree). Cells tend to align along the longitudinal direction of the striped pattern, which is perpendicular to the centrifugal force direction. The tendency continues for 24 hours after the centrifugal force stimulation. The number of cells decreases after the centrifugal force stimulation hysteresis: no cell is on the area of the striped pattern of 90 degree at 6 hours, and at 18 hours after the centrifugal force stimulation hysteresis.

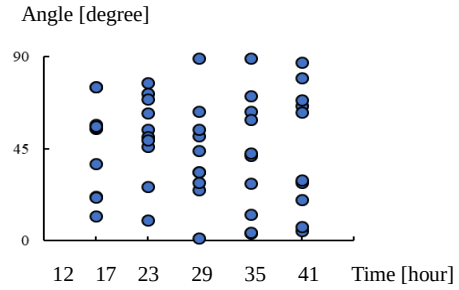

Fig. 2: Angle of major axis of each cell on flat surface at 0 G.

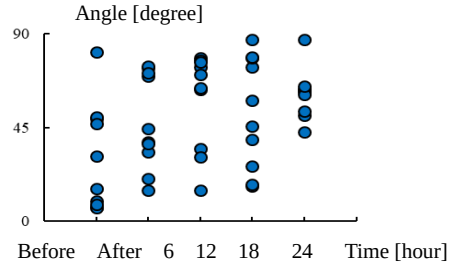

Fig. 3a: Angle between major axis of each cell and tangential force of 50 G on flat surface: before, after, 6h, 12 h, 18 h, 24 h.

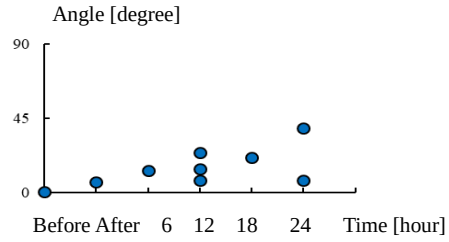

Fig. 3b: Angle between major axis of each cell and tangential force of 50 G on striped pattern of 0 degree.

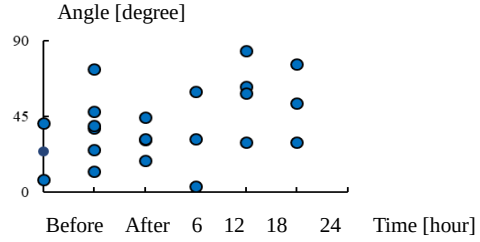

Fig. 3c: Angle between major axis of each cell and tangential force of 50 G on striped pattern of 45 degree.

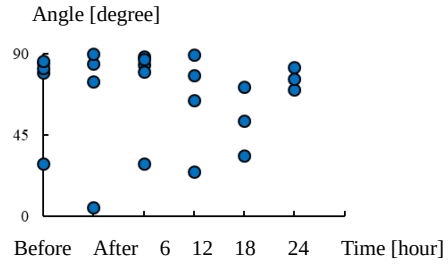

Fig. 3d: Angle between major axis of each cell and tangential force of 50 G on striped pattern of 90 degree.

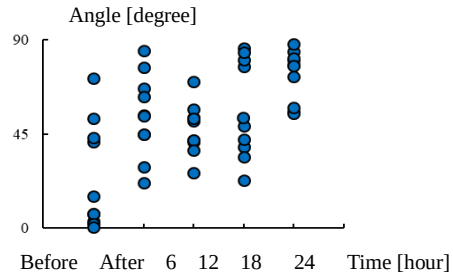

Fig. 4a: Angle between major axis of each cell and tangential force of 100 G on flat surface.

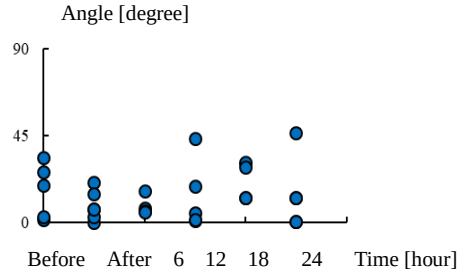

Fig. 4b: Angle between major axis of each cell and tangential force of 100 G on striped pattern of 0 degree.

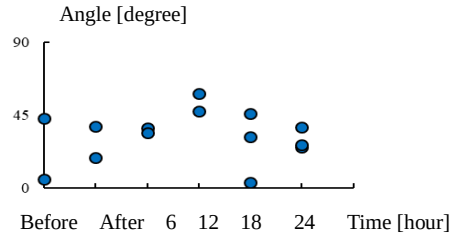

Fig. 4c: Angle between major axis of each cell and tangential force of 100 G on striped pattern of 45 degree.

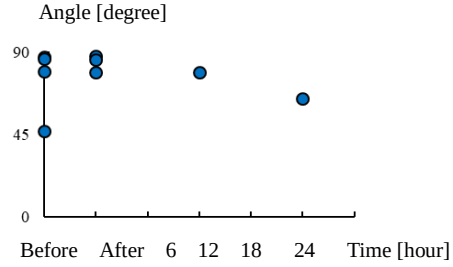

Fig. 4d: Angle between major axis of each cell and tangential force of 100 G on striped pattern of 90 degree.

Figs. 5, 6 and 7 show the shape index (*P*). Fig. 5 shows the shape index of each cell without centrifugal force stimulation (control study) on the flat surface. Cells are elongated within 12 hours after adhesion to the scaffold. Fig. 6 shows the shape index of each cell at 50 G on each micro pattern: 0 degree (Fig. 6b), 45 degrees (Fig. 6c), and 90 degrees (Fig. 6d), respectively. Fig. 6a shows data on the flat surface. Cells tend to be rounded after centrifugal force stimulation hysteresis.

Fig. 7 shows the shape index of each cell at 100 G on each micro pattern: 0 degree (Fig. 7b), 45 degrees (Fig. 7c), and 90 degrees (Fig. 7d), respectively. The number of rounded cells (small value of *P*) on the ridges decreases immediately after centrifugation (Fig. 7b & 7c). Cells tend to be elongated to the direction of centrifugal force. The tendency is relieved in 12 hours after stopping of the centrifugal force stimulation. The elongation tendency is apparent, when the longitudinal direction of the micro ridge is parallel to the direction of centrifuge (Fig. 7b). The number of cells decreases after the centrifugal force stimulation hysteresis (Fig. 7d).

Each cell extends along the striped pattern more frequently in 6 hours after centrifugal force stimulation. The tendency decreases in 12 hours after centrifugal force stimulation. At proliferation, each cell tends to elongate to the direction perpendicular to the hysteretic tangential force direction.

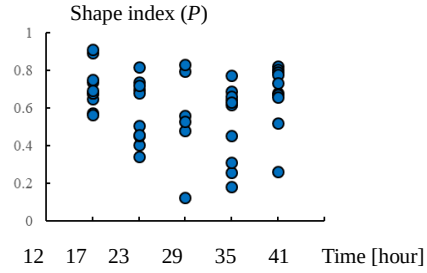

Fig. 5: Shape index (*P*) of each cell on flat surface at 0 G.

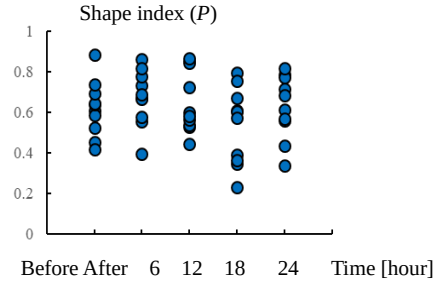

Fig. 6a: Shape index (*P*) of each cell on flat surface after 50 G.

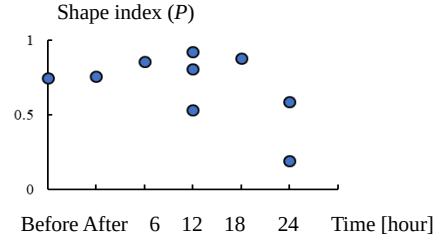

Fig. 6b: Shape Index (*P*) of each cell on 0 degree after 50 G.

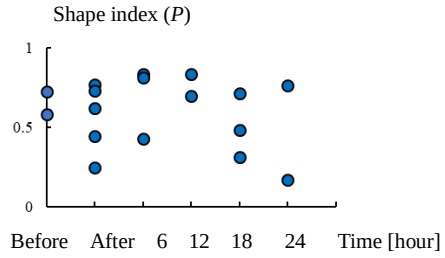

Fig. 6c: Shape Index (*P*) of each cell on 45 degree after 50 G.

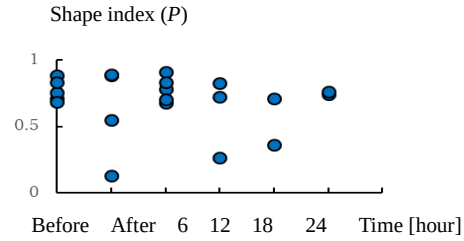

Fig. 6d: Shape Index (*P*) of each cell on 90 degree after 50 G.

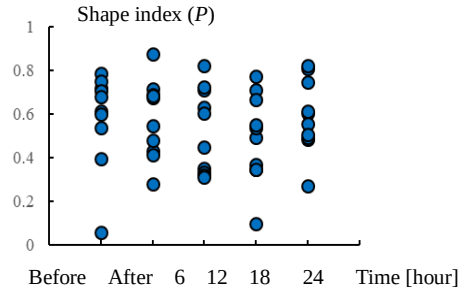

Fig. 7a: Shape index (*P*) of each cell on flat surface after 100 G.

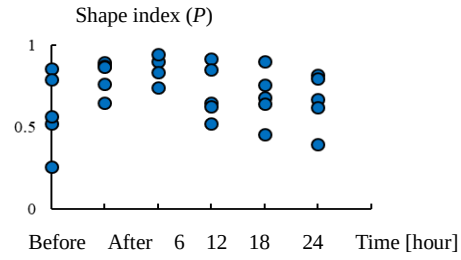

Fig. 7b: Shape Index (*P*) of each cell on 0 degree after 100 G.

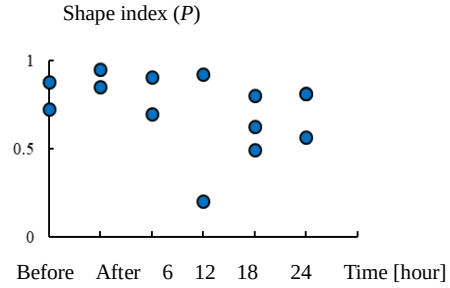

Fig. 7c: Shape Index (*P*) of each cell on 45 degree after 100 G.

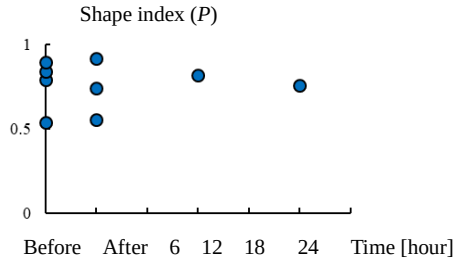

Fig. 7d: Shape Index (*P*) of each cell on 90 degree after 100 G.

4. DISCUSSION

The gravity in the fluid is reduced by the buoyancy. Measurement of the density of cells by Phthalate ester method shows that the density of MC3T3-E1 is between 1.06×10^3 kg/m^3 and 1.07×10^3 kg/m^3. When the cells floating in the medium of the density of 1.00×10^3 kg/m^3, the effective centrifugal force ratio calculated from the difference of two density is 6.5 G at centrifuge of 100 G. During centrifugation, the direction of the force field slightly tilts from horizontal direction (vertical at the scaffold in the present study) because of the gravity (1 G) of the earth. In the case of centrifuge of 5 G, the shift of the angle is 11 degree. The time lapse image of every ten minutes is effective to trace the movement of each cell. The response of the cell depends on the direction of the hyper-gravitational field. Each cell changes its own direction during migration. The migration depends on the morphology of the scaffold surface [8]. When a cell cannot keep adhesion under stimulation, the shape index (*P*) approaches to "0" to be a sphere. Each cell deforms and migrates randomly. When the cell rounds, the contact between the cell and the scaffold decreases. The cell easily exfoliates from the scaffold at the small contact area. After the cell rounds at proliferation, alignment of cells along the longitudinal direction of the striped pattern is relieved. The average cell cycle of proliferation is 24 hours. The term for 41 hours from seeding is selected in the present experiment. The elongated cell, on the other hand, tends to align along the longitudinal direction of the striped pattern of ridges on the scaffold surface. The cell on the striped pattern not parallel to the centrifugal force stimulation (at 45 degree, and at 90 degree) hardly makes contact to the scaffold surface at 100 G. The hysteresis of centrifugation might make each cell to increase the contact area with the surface of the scaffold. Each cell tends to align along the pattern on the scaffold surface at the higher tangential force field (100 G) than at the lower tangential force field (50 G). Data scatter in Fig. 3c than in Fig 4c. The variation was made on the position (radius) of cell culture in the same rotor of the centrifuge, to test in the different gravitational environment simultaneously [6]. The main reason why number of cells decrease in Fig. 4d and in Fig. 7d is not exfoliation, but cells tend to migrate to the direction to keep away from the area of the stripes of 90 degree (perpendicular to the centrifugal force direction). Cells passively follow the direction in the strong tangential force field. Immediately after the centrifugation, cell might start to show active response to the mechanical stimulation. The mild tangential force field induces the active reflection of myoblasts to tilt perpendicularly to decrease internal force of the cells. The cell shows adaptation against stimulation. The stimulation leaves hysteresis in the cell. The hyper-gravitational stimulation governs the behavior of the

cell after stimulation. The number of cells, which do not align to the longitudinal direction of ridges, tends to decrease by the hyper-gravitational stimulation. In the previous study, the tendency of the C2C12 to align along the longitudinal direction of ridges is enhanced on the ridge perpendicular to the direction of the excess gravity, after centrifuge [7]. To align the direction 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 [8]. The average diameter of the floating cell in the medium is 20 μm. Both the low height and the small width of the ridge may not disturb the migration of cells over the ridge. The behavior of each cell can be easily traced with the aid of the micro striped pattern on the scaffold as the marker. To apply continuous uniform mechanical stimulation to the cells, centrifugal force is used in the present study. The effect of mechanical field on orientation and deformation of several kinds of cells was studied using centrifuge *in vitro* in the previous studies [1, 6, 7, 9–12]. The response of biological system to the microgravity field has been studied using a space satellite [13–15]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [6]. The muscle tissue might decrease in the micro gravitational field [15]. The muscle tissue might increase, on the other hand, in the hypergravity [1, 16]. The previous study shows that the hyper-gravitational field thickens the myotubes *in vitro* [1]. 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 on MC3T3-E1 (mouse osteoblast precursor cell) cultured at the surface of the scaffold with the micro striped pattern has been studied *in vitro*. The lines of parallel micro quadrangular ridges (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 about the angle between the longitudinal direction of the ridge and the direction of the tangential force field: 0 degree, 45 degrees, and 90 degrees. To apply the tangential force field (50 G, 100 G) on the surface of the scaffold, the plate was set in the tube in a conventional centrifugal machine placed in an incubator. The experimental results show that the cell elongates to the longitudinal direction of the striped pattern by the hysteresis of the exposure to the tangential force field more frequently at 100 G than at 50 G. Some cells are rounded at proliferation, and relieved to align along the longitudinal direction of the striped pattern. Cells tend to elongate at the striped pattern parallel to the tangential force, immediately after the centrifugal stimulation of 100 G.

REFERENCES

[1] S. Hashimoto, H. Hino and T. Iwagawa, “Effect of Excess Gravitational Force on Cultured Myotubes in Vitro”, **Journal of Systemics, Cybernetics and Informatics**, Vol. 11, No. 3, 2013, pp. 50–57.

[2] M. Ochiai, S. Hashimoto and Y. Takahashi, “Effect of Flow Stimulation on Cultured Osteoblast”, **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 156–161.

[3] H. Hino, S. Hashimoto, Y. Takahashi and M. Ochiai, “Effect of Shear Stress in Flow on Cultured Cell: Using Rotating Disk at Microscope”, **Journal of Systemics Cybernetics and Informatics**, Vol. 14, No. 4, 2016, pp. 6–12.

[4] S. Hashimoto, Y. Takahashi, H. Hino, R. Nomoto and T. Yasuda, “Micro Hole for Trapping Flowing Cell”, **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 114–119.

[5] Y. Takahashi, S. Hashimoto, Y. Hori and T. Tamura, “Sorting of Cells Using Flow Channel with Oblique Micro Grooves”, **Proc. 22nd World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2018, pp. 138–143.

[6] T. Tamura, H. Hino, S. Hashimoto, H. Sugimoto and Y. Takahashi, “Cell Behavior After Stimulation of Excess Gravity”, **Proc. 21st World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2017, pp. 263–268.

[7] T. Tamura, H. Hino and S. Hashimoto, “'Behavior of Cell Cultured on Micro Striped Pattern after Stimulation of Excess Gravity”, **Proc. 22nd World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2018, pp. 144–149.

[8] H. Hino, S. Hashimoto and F. Sato, “Effect of Micro Ridges on Orientation of Cultured Cell”, **Journal of Systemics, Cybernetics and Informatics**, Vol. 12, No. 3, 2014, pp. 47–53.

[9] T. Tamura, S. Hashimoto, H. Hino, T. Ito and Y. Endo, “Tracings of Orientation of Cell on Scaffold with Micro Striped Pattern after Stimulation of Vertical Excess Gravity”, **Proc. 22nd World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2018, pp. 150–155.

[10] G. Ciofani, L. Ricotti, J. Rigosa, A. Menciassi, V. Mattoli and M. Monici, “Hypergravity Effects on Myoblast Proliferation and Differentiation”, **Journal of Bioscience and Bioengineering**, Vol. 113, No. 2, 2012, pp. 258–261.

[11] H. Hino, H. Sugimoto, Y. Takahashi, S. Hashimoto and S. Miura, “Behavior of Cells in Excess Gravitational Field: Using Centrifuge”, **Proc. 20th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2016, pp. 123–128.

[12] H. Hino, H. Sato, S. Hashimoto and Y. Takahashi, “Effect of Excess Gravitational Force and Electric Pulse Field on Myoblast”, **Proc. 19th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2015, pp. 258–263.

[13] E.R. Morey and D.J. Baylink, “Inhibition of Bone Formation during Space Flight”, **Science**, Vol. 201, No. 4361, 1978, pp. 1138–1141.

[14] M. Hughes-Fulford and L.M. Lewis, “Effect of Microgravity on Osteoblast Growth Activation”, Experimental **Cell Research**, Vol. 224, No. 1, 1996, pp. 103–109.

[15] R. Lalani, S. BhaSIn, F. Byhower, R. Tarnuzzer, M. Grant, R. Shen, S. Asa, S. Ezzat and F.N. Gonzalez-Cadavid, “Myostatin and Insulin-like Growth Factor-I and -II Expression in the Muscle of Rats Exposed to the Microgravity Environment of the Neuro Lab Space Shuttle Flight”, **Journal of Endocrinology**, Vol. 167, No. 3, 2000, pp. 417–428.

[16] A. Tschopp and A. Cogoli, “Hypergravity Promotes Cell Proliferation”, **Experientia**, Vol. 39, No. 12, 1983, pp. 1323-1329.