Effect of Aspect Ratio of Checkered (Ichimatsu) Convexo-concave Micro-pattern on Orientation of Cultured Cells

Kenta SUGIMOTO, Yusuke TAKAHASHI, Haruka HINO, Shigehiro HASHIMOTO

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

ABSTRACT

The effect of the pattern of micro ridges on orientation of the cell culture has been studied *in vitro*. The checkered (Ichimatsu) convexo-concave pattern has been designed with micro quadrangular prisms in the square area of 1 mm × 1 mm on a disk of glass for a scaffold by the lithography technique. Each prism has the following dimension: 0.01 mm length, and 0.005 mm (or 0.001 mm) height. Variation has been made on the width of the prism: 0.005 mm, 0.008 mm, and 0.01 mm. The variation of the width makes the variation on the aspect ratio of the top rectangular surface of the prism: 1, 1.25, and 2. Three kinds of cells were used in the test: C2C12 (mouse myoblast cell line), Neuro-2a (a mouse neural crest-derived cell line), and Hepa1-6 (mouse hepatoma cell line). Cells were seeded on the micro pattern, and incubated for 21 days in the Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% penicillin/ streptomycin. The cells were observed with a phase contrast microscope. The experimental results show that the orientation of myotubes can be controlled by the aspect ratio of the checkered micro convexo-concave pattern of the surface of the scaffold.

Keywords: Biomedical Engineering, Cell Culture, Checkered Micro pattern and Orientation.

1. INTRODUCTION

A biological cell adheres, migrates, rotates, and deforms on the scaffold. These behaviors of cell depend on the micro morphology of the scaffold [1-10]. The cell might be sensitive to the morphology of the similar dimension to itself at the scaffold. The photolithography technique is available to make the micro patterns on the scaffold of the cell culture. The previous study showed that the orientation of myoblast depends on the height of the micro ridges [1].

Several methodologies have been clinically applied to regenerative medicine. The acceleration technique for proliferation, orientation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [11-13]. The behavior of a cell depends on several factors: mechanical [14, 15], electrical [12, 13, 16], and magnetic stimulations [17]. The effect of stimulations on the cell varies with the kind of cells. Control methodology for proliferation, orientation and differentiation of cells would be applied to the regenerative tissue technology.

In the present study, effects of the aspect ratio of the checkered pattern of micro quadrangular prisms on orientation of the cell culture have been studied *in vitro*.

2. METHODS

Micro Pattern

The "Ichimatsu" checkered convexo-concave pattern has been designed with micro quadrangular prisms at the square area of 1 mm × 1 mm on a disk of glass for a scaffold by the lithography technique (Fig. 1). Each prism has the following dimension. The length of the top rectangular surface is 0.01 mm length. Variation has been made on the width of the top square of the prism: 0.005 mm, 0.008 mm, and 0.01 mm. The variation of the width makes the variation on the aspect ratio of the top rectangular surface of the prism: 1, 1.25, and 2. The arctangent of the each ratio is 45, 51, and 63 degree. Variation has been made on the height of the prism using two kinds of photomasks: 0.005 mm by the mask A, and 0.001 mm by the mask B.

Each pattern is drawn in the square area of 0.5 mm × 0.5 mm, which is the quarter part of the square area of 1.0 mm × 1.0 mm. The square area is surrounded by a smooth surface without pattern as control.

Photomasks were used to trace the micro checkered pattern on the mold. The photomasks were made by two kinds of etching process: wet and dry.

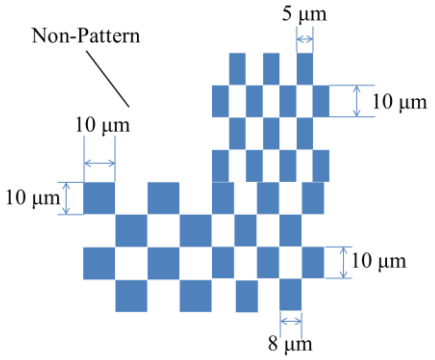

Fig. 1: Three kinds of checkered micro-pattern. Aspect ratio of the top rectangular surface of the prism: 1 (lower left), 1.25 (lower right), 2 (upper right), and flat (upper left).

Photomask A by Wet Etching

The borosilicate glass (Tempax) disk (35 mm diameter, 1mm thickness) was used for the base of the photomask (Fig. 2). After hydrophilization by the oxygen (30 cm^3/min) plasma ashing at 100 W for ten minutes by RIE (FA-1, Samco International, Kyoto, Japan), chromium was deposited on the surface with 300 nm thickness by the sputtering equipment of L-210S-FH (Canon Anelva Corporation) for three minutes. To improve affinity between chromium and photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the disk at 3000 rpm for 30 s with a spin coater. The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the chromium at 5000 rpm for 30 s with the spin coater. The photoresist was baked at the hotplate in two processes: at 338 K for one minute, and 368 K for three minutes.

The checkered pattern was drawn on the mask with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern on the photomask with the laser drawing system, the parameters were selected as follows: the voltage of 2.3 V, the velocity of 0.08 mm/s, the acceleration of 0.5 mm/s^2. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for five minutes, rinsed with the distilled water, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan) (Fig. 3a).

The chromium coating was etched in etchant for chromium TW (Nihon Kagaku Sangyo Co., Ltd., Tokyo) for twenty minutes. To remove the residual layer of OFPR-800LB, the mask was dipped in acetone for two minutes, rinsed with the distilled water, and dried by the spin-dryer (Fig. 3b).

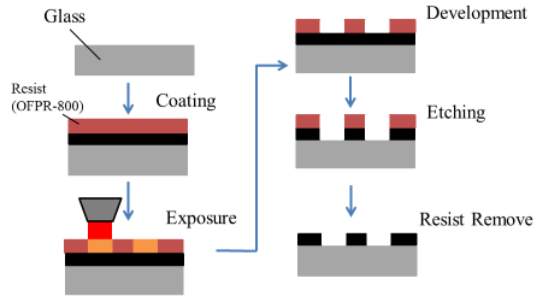

Fig. 2: Photomask by photolithography.

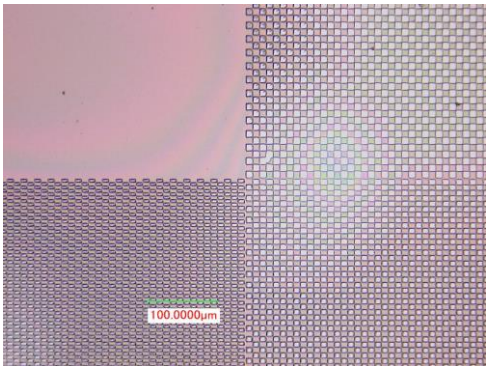

Fig. 3a: Photomask A before etching. Dimension from left to right is 0.7 mm.

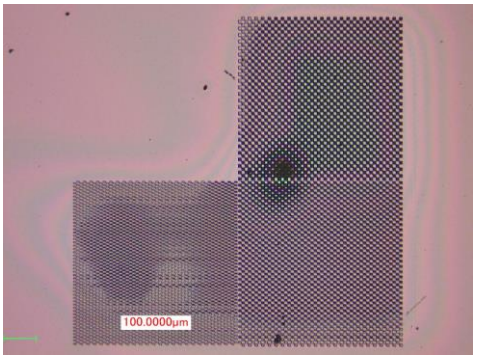

Fig. 3b: Photomask A after wet etching. Dimension from left to right is 1.4 mm.

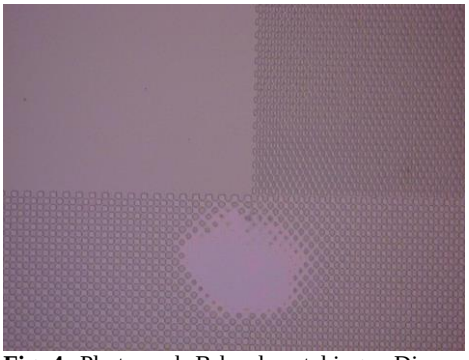

Fig. 4: Photomask B by dry etching. Dimension from left to right is 0.7 mm.

Photomask B by Dry Etching

After hydrophilization by the oxygen plasma ashing by RIE, titanium was deposited on the surface with 150 nm thickness on the surface of the borosilicate glass by the sputtering equipment for eight minutes. To improve affinity between titanium and photoresist material, HMDS was coated. The positive photoresist material of OFPR-800LB was coated on the glass. The checkered pattern was drawn on the mask with the laser drawing system.

To control the dimension of the pattern on the photomask with the laser drawing system, the parameters were selected as follows: the voltage of 2.3 V, the velocity of 0.08 mm/s, the acceleration of 0.5 mm/s^2.

The photo-resist was developed with tetra-methyl-ammonium hydroxide. The glass was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan). For etching, the gas of SF_6 (50 cm^3/min at 1013 hPa) with Ar (50 cm^3/min at 1013 hPa) was applied at 100 W at 4 Pa for five minutes (Fig. 4).

To remove the residual layer of OFPR-800LB, the mask was dipped in acetone for two minutes, rinsed with the distilled water, and dried by the spin-dryer.

Scaffold with Micro Pattern

The borosilicate glass (Tempax) disk (35 mm diameter, 1mm thickness) was used for the base for micro quadrangular prisms, after cleaning and hydrophilization. The negative photoresist material of high viscosity (SU8: Micro Chem Corp., MA, USA) was coated on the glass at 3000 rpm (7000 rpm on B) for one minute with a spin coater. The photoresist baked in the oven

at 368 K for five minutes. The photomask was adhered on the surface of SU8-10, and the photoresist was exposed to the UV light through the mask in the mask aligner (M-1S, Mikasa Co. Ltd., Japan) for 250s (8 s on mask B) (Fig. 5A). The photoresist was developed with SU8 developer (Nippon Kayaku Co., Ltd, Tokyo, Japan) for six minutes to make micro prisms. The glass with the micro pattern was rinsed with isopropyl alcohol for two minutes, and dried by spin-dryer.

The dimension of the micro pattern of the mold was measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan) (Fig. 5B). The morphology along the transverse lines of ridges was traced.

A disk of polydimethylsiloxane (PDMS), which has a donut shape (35 mm outer diameter, 3 mm thickness) with a hole of 10 mm diameter (culture area of 3.1 cm^2), was made for the peripheral wall of the dish (Fig. 6).

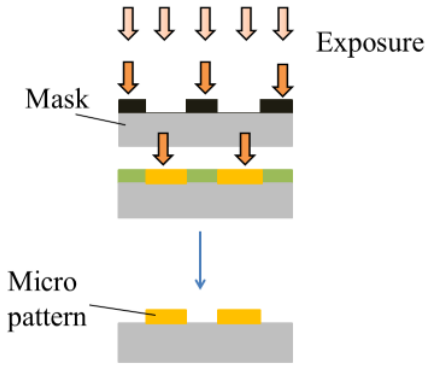

Fig. 5A: Micro pattern by photolithography.

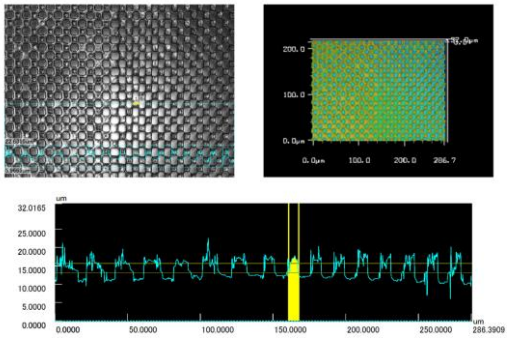

Fig. 5B: Tracing of scaffold by photomask A (by laser microscope).

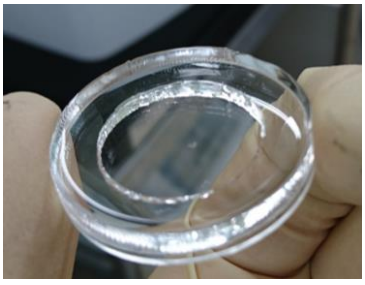

Fig. 6: Culture dish (35 mm diameter) with micro pattern (center).

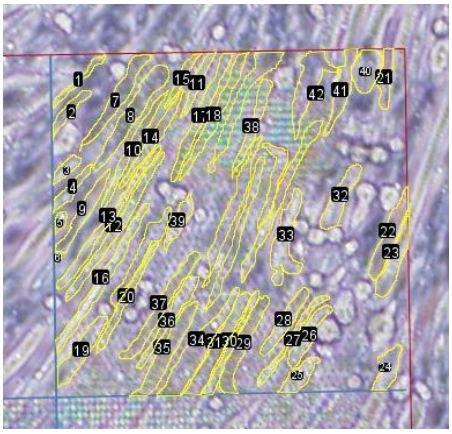

Fig. 7: Tracings of contour of each myotube. Dimension from left to right is 0.7 mm.

After the disk of Tempax glass was enclosed with a peripheral wall of polyimide, degassed 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). The volume ratio of PDMS to curing agent is ten to one. PDMS was baked at 373 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd).

The culture plate was exposed to the oxygen gas in a reactive ion etching system (FA-1, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing).

Cell Culture

Three kinds of cells were used in the test: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), Neuro-2a (a mouse neural crest-derived cell line), and Hepa1-6 (mouse hepatoma cell line of C57L mouse).

Cells were seeded on the micro pattern at the density of 1000 cells/cm^2 (2000 cells/cm^2 for pattern B), and incubated (< 21 days) in the Dulbecco's Modified Eagle Medium. The medium contains 10% fetal bovine serum and 1% penicillin/ streptomycin. In the case of Neuro-2a, Retinoic Acid was added to the medium to induce differentiation. The concentration of Retinoic Acid in the medium is adjusted to 20 μM.

The culture dish was kept in the incubator to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent. The medium was changed every two days. The cells were continuously observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture.

On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid (Fig. 7). The angle between the longitudinal axis of each cell and the shorter side of the square of the checkered pattern was measured.

3. RESULTS

Table 1 shows the dimension of height measured by the laser microscope (Fig. 5B). At the manufactured micro pattern A, there are slits between the micro quadrangular prisms, because rectangles are bridged together at the photomask A (Fig. 3b). At the manufactured micro pattern B, on the other hand, there

are bridges between the micro quadrangular prisms, because each rectangle is separated island at the photomask B (Fig. 4).

Table 1: Dimension of the height of the prism, used in each test of three kinds of cells: micro pattern with mask A and B.

Cell	mm	
	Pattern A	Pattern B
Neuro-2a	0.005	
C2C12	0.005	0.001
Hepa1-6		0.002

Neuro-2a extends the neurite along the slit between the micro quadrangular prisms (Fig. 8). The proliferation of Neuro-2a decreases at the micro pattern. The checkered pattern does not affect orientation of Hepa1-6 (Fig. 9).

On the checkered convexo-concave micro pattern, myoblasts (Figs. 10a & 11a) were differentiated to myotubes (Figs. 10b & 11b). At the micro pattern A, the myotubes tend to make orientation along the diagonal line of slit between the micro quadrangular prisms (Fig. 10c). At the micro pattern B, on the other hand, the myotubes tend to make along the longitudinal direction of the rectangular top surface of the micro quadrangular prisms (Fig. 11b).

Fig. 12 shows the angle between the longitudinal axis of each myotube and the shorter side of the square of the checkered pattern measured at the image of Fig. 10c.

Data are arranged in ascending order in Fig. 12a. Data will make flat section at the large frequency. At the random frequency, data will form a simple rising straight line. Every group of data at each micro pattern shows the character of the inverse "S", which correspond to high frequency around the mean value.

Fig. 12b shows the relation between the mean longitudinal axis direction of myotube and the angle of the diagonal line of each rectangle. The angle of zero degree indicates direction to shorter side of the rectangle of checkered pattern. Fig. 12b shows that the angle of the myotube is proportional to that of diagonal line of the micro rectangle.

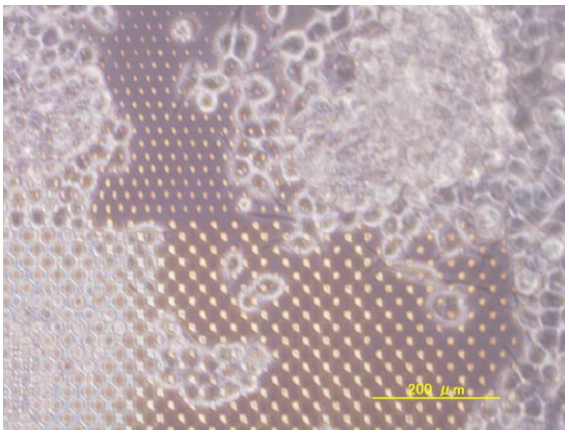

Fig. 8: Neuro-2a cultured on micro pattern A for 6 days. Dimension from left to right is 0.9 mm.

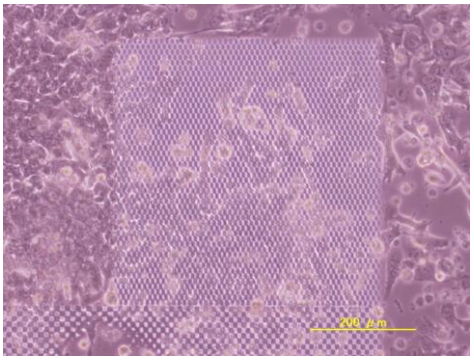

Fig. 9a: Hepa1-6 cultured on micro pattern B for 7 days. Dimension from left to right is 1 mm.

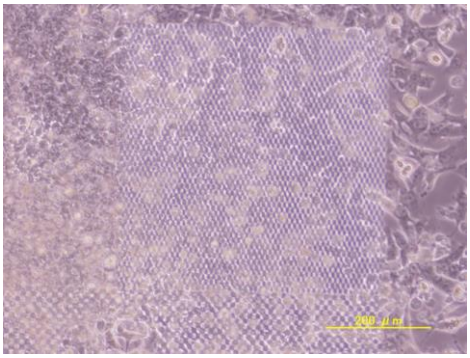

Fig. 9b: Hepa1-6 cultured on micro pattern B for 9 days. Dimension from left to right is 1 mm.

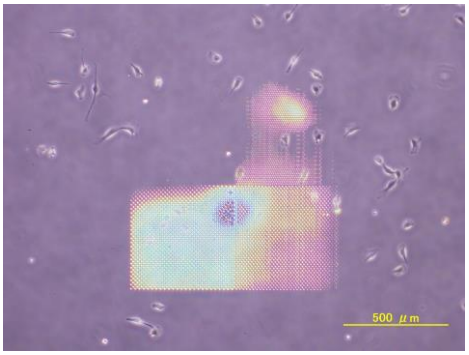

Fig. 10a: C2C12 cultured on micro pattern A for 1 day. Dimension from left to right is 2 mm.

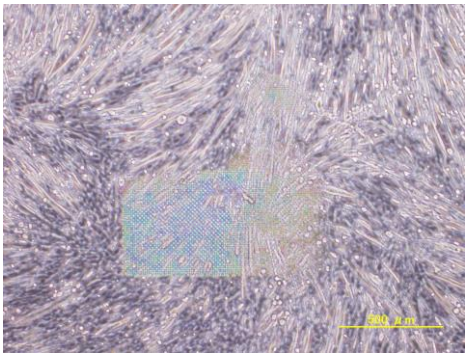

Fig. 10b: C2C12 cultured on micro pattern A for 12 days. Dimension from left to right is 2 mm.

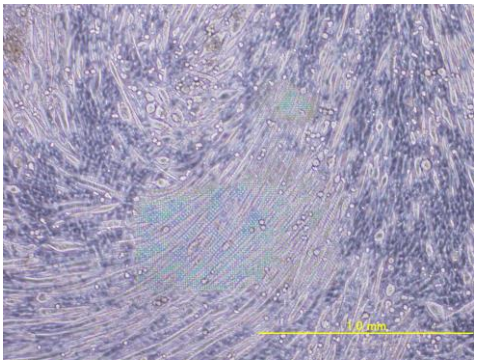

Fig. 10c: C2C12 cultured on micro pattern A for 20 days. Dimension from left to right is 2 mm.

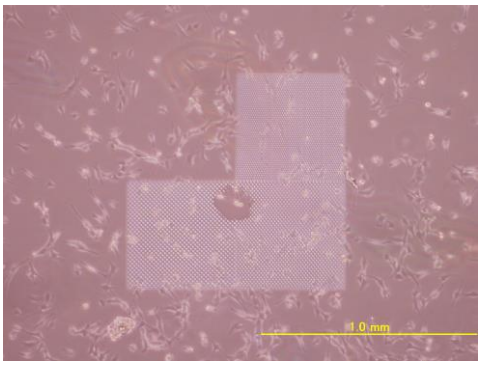

Fig. 11a: C2C12 cultured on micro pattern B for 1 day. Dimension from left to right is 2 mm.

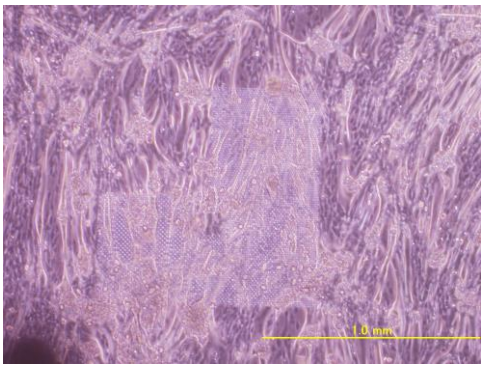

Fig. 11b: C2C12 cultured on micro pattern B for 10 days. Dimension from left to right is 2 mm.

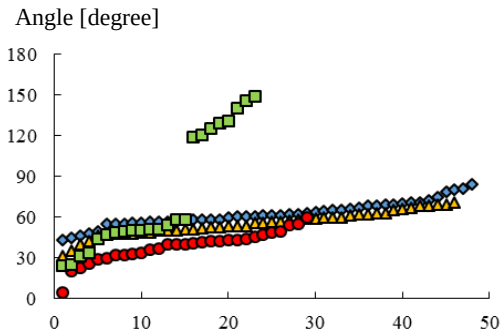

Fig. 12a: Angle of longitudinal axis of myotube: aspect ratio 1 (circle), 1.25 (triangle), 2 (rhombus), and flat (square)

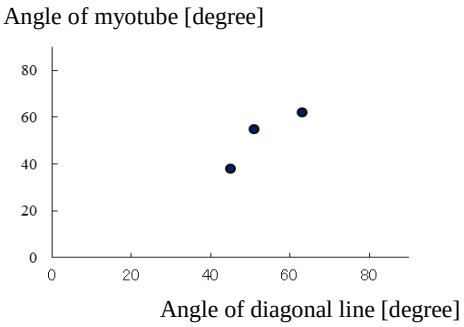

Fig. 12b: Mean longitudinal axis direction of myotube vs. diagonal line of each square. Angle of zero degree indicates direction to shorter side of the square of checkered pattern.

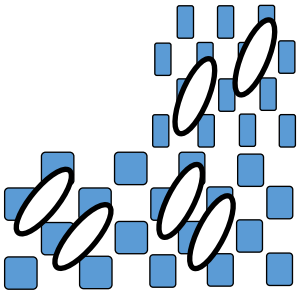

Fig. 13a: Orientation of cell on checkered pattern A.

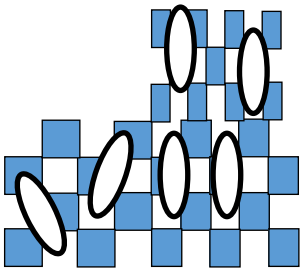

Fig. 13b: Orientation of cell on checkered pattern B.

4. DISCUSSION

On the manufactured pattern A, the micro slits are left between the micro quadrangular prisms (Fig. 13a). Myotubes might make orientation along the slit on checkered pattern A. On the manufactured pattern B, on the other hand, the micro quadrangular prisms are connected together (Fig. 13b). The myotube might make orientation along the longitudinal direction of the rectangular top surface of the micro quadrangular prisms. C2C12 migrates and extends along the longitudinal axis of the ridges [1].

The height of the micro quadrangular prism is higher at the pattern A than that at the pattern B. The height of the pattern B might have not enough height to make orientation of cells.

The effect of the height of micro ridges on the orientation of C2C12 was studied in the previous study [1]. The experimental results show that myoblasts adhere on the top of the ridge and align to the longitudinal direction of the micro ridges with the height between 0.00015 mm and 0.0025 mm.

The height (H) of the micro ridge is selected to be 0.001 mm < H < 0.005 mm in the present study.

The orientation in the surrounding area might follow that of the rectangular pattern. The orientation of myotubes developed at day 20 of culture (Fig. 10c) after day 12 of culture (Fig. 10b). Myotube has tendency to make orientation following direction of neighbor myotube [11].

On the Ichimatu pattern of 0.005 mm height, observation of cells is not easy because of the light scattering through the micro pattern.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The previous study shows that electrical stimulation enhances differentiation of muscle cells [13, 16]. The previous studies show that a mechanical field, on the other hand, governs behavior of cells. The shear flow governs the orientation of endothelial cells [11, 14]. Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [15]. The mechanical stimulation can decrease proliferation of cells [15]. The mechanical stress also exfoliates several cells [14], which makes vacancy around the adhered cell. The differentiation might be optimization of cells to the changing environment. The mechanical stress can accelerate differentiation of C2C12 into myotubes.

5. CONCLUSION

Effects of the pattern of micro ridges on orientation of the cell culture have been studied *in vitro*. The checkered (Ichimatsu) convexo-concave pattern has been designed with micro quadrangular prisms in the square area of 1 mm × 1 mm on a disk of glass for a scaffold by the lithography technique. Each prism has the following dimension: 0.01 mm length, and 0.005 mm (or 0.001 mm) height. Variation has been made on the width of the prism: 0.005 mm, 0.008 mm, and 0.01 mm. Three kinds of cells were used in the test: C2C12 (mouse myoblast cell line), Neuro-2a (a mouse neural crest-derived cell line), and Hepa1-6 (mouse hepatoma cell line). The experimental results show that the orientation of myotubes can be controlled by the aspect ratio of the checkered micro convexo-concave pattern of the surface of the scaffold.

6. ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Strategic Research Foundation at Private Universities from the Japanese Ministry of Education, Culture, Sports and Technology.

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