Effect of Micro Ridges on Orientation of Cultured Cell

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ABSTRACT

The effect of micro ridges on orientation of cultured cells has been studied *in vitro*. Several patterns of micro ridges have been fabricated on a transparent polydimethylsiloxane disk with the photo lithography technique. The ridges consist of several lines of rectangular column: the width of 0.003 mm, the interval of 0.007 mm. Variation has been made on the height of the ridge between 0.0003 mm and 0.0035 mm. C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was cultured on the disk with the micro ridges for one week and was observed with an inverted phase contrast microscope. The experimental results show that cells adhere on the top of the ridge and align to the longitudinal direction of the micro ridges with the height between 0.0015 mm and 0.0025 mm.

Keywords: Biomedical Engineering, Cell Culture, C2C12, Lithography, Micro Ridge and Polydimethylsiloxane.

1. INTRODUCTION

Biological cells respond to various environmental factors, such as electric [1], magnetic [2] and mechanical [3-5] fields. These factors affect cells behaviors: migration, deformation, orientation, proliferation, differentiation and secretion of extracellular matrix. Through these processes, the various environmental factors govern the configuration of the biological tissue.

Cell culture technique has been developed and several methodologies have been clinically applied to regenerative medicine [6]. The acceleration technique for orientation and proliferation of cells has been studied to make a biological tissue *in vitro* or *in vivo* [3, 6]. Control methodology for behavior of cells would be applied to regenerative tissue technology.

The morphology of the surface of the scaffold might affect the orientation of cells and might govern organization of cells [7, 8]. Several methods have been applied to make orientation of cell culture in the previous studies: fibers [9] and grooves [10-12].

In the previous studies, the orientation of cells was controlled by sandwiching the cells between walls: in grooves, or in capillaries. The wall prohibits extension of cells, and regulates the direction of cells. A capillary of sub-millimeter diameter was used to make a cylindrical tissue in the previous study. In such kinds of the wider interval between walls than dimension of the cell of micrometers, direction of each cell might not be controlled.

The cell, on the other hand, might sense the direction of micromorphology. The smaller interval of ridges may control the direction of the single cell [13]. In the present study, the cell has been cultured on the linear ridges.

The photo lithography technique is effective to make micro patterns on the surface of the plate for cell culture. In the present study, the effect of micro ridges on orientation of cultured cells has been studied *in vitro*.

2. METHODS

Micro Ridges
Several (between one and ten) parallel lines of micro ridges have been made on a disk of transparent polydimethylsiloxane (PDMS). The width (*W*), the interval (*I*) and the length (*L*) of the rectangular ridge (Figs. 3, 15) are around 0.003 mm, 0.007 mm, and 2 mm respectively. Variation has been made on the height (*H*) of the ridge: 0.0003 mm, 0.0007 mm, 0.0015 mm, 0.0025 mm, and 0.0035 mm.

A silicon wafer (Type P, Matsuzaki Seisakusyo, Co., Ltd., Tokyo, Japan) is used for a surface mold for the disk (Fig. 1) in the photo lithography process (Fig. 2). The diameter and the thickness of the wafer are 50 mm and 0.30 mm, respectively.

The surface of the wafer was cleaned by the following process. The surface was exposed to the oxygen gas in a reactive ion etching system (FA-1, Samco Inc., Kyoto) for five minutes: (the oxygen plasma ashing). It was cleaned in an ultrasonic cleaner with 2-propanol for five minutes, with the hydrogen peroxide solution for five minutes, and with the ultrapure water for five minutes. Then, the wafer was dried on the hot plate (AHP-300, Asahi-rika, Chiba, Japan) at 373 K for five minutes.

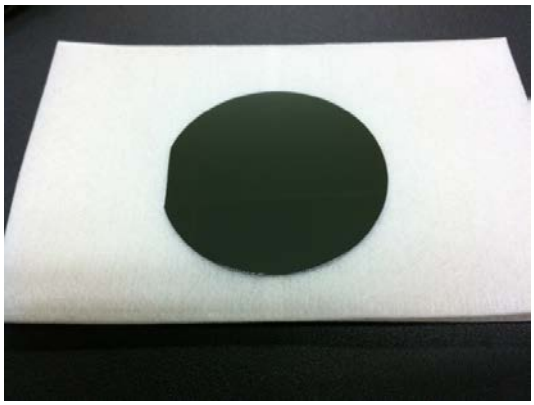

Fig. 1: Silicon wafer for mold (diameter: 50 mm).

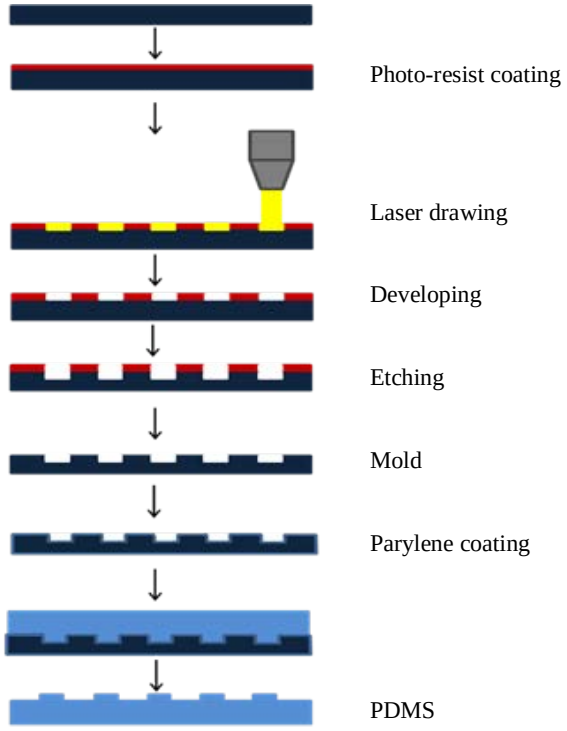

Fig. 2: Photo lithography process.

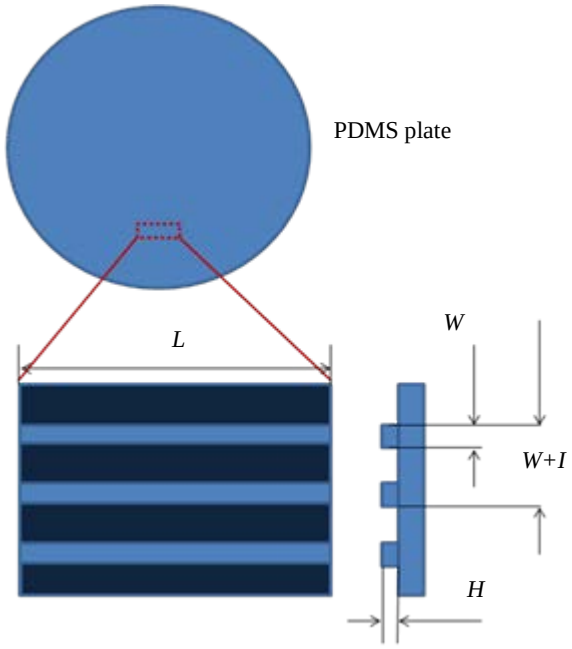

Fig. 3: Typical location of micro ridges (magnified in bottom) on the plate of PDMS plate.

The photo-resist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the wafer with 0.002 mm thick at 5000 rpm with a spin coater. The photo-resist was baked in an oven (DX401, Yamato Scientific Co., Ltd, Tokyo, Japan) at 383 K for 90 seconds.

The pattern of lines was drawn on the wafer with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the lines of the mold with the laser drawing system, the parameters were selected as follows: the wave length 408 nm, and the power 20 mW. After the drawing, the photo-resist was baked again in an oven at 393 K for five minutes.

The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for 90 seconds. The wafer was rinsed with the distilled water for 30 seconds. To increase the adhesiveness of the coating, the wafer was baked at 393 K for five minutes.

The wafer was etched with the plasma gas using the reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan) to make lines of the micro groove. The alternative switching mode between $C4F_8$ gas and SF_6 gas was applied on the disk.

To exfoliate the residual photo-resist material from the surface, the wafer was exposed to the oxygen gas at power of 50 W with flow of 30 milliliter per minute for five minutes: (the oxygen plasma ashing).

The dimension of the micro grooves of the mold was measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). Each mold has several groups of ridges dispersed on the disk. The number of lines of ridge varies with each group. Each mold has its own unified height of the ridges.

The surface of the wafer with micro pattern was coated with 0.001 mm thickness of parylene in the parylene coater (PDS-2010, Speciality Coating Systems, Indianapolis, USA).

After the wafer was enclosed with a peripheral wall of polyimide, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corporation) with the curing agent (Dow Corning Corporation) was poured on the wafer. The volume ratio of the curing agent is ten percent of PDMS. After degassing, PDMS was baked at 383 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd).

The baked disk of PDMS is exfoliated from the mold, and sterilized in an autoclave. The disk with the micro ridges at several positions was cut into the plate of 35 mm diameter (Fig. 3) to be set at the bottom of the dish of six wells. The plate was exposed to the oxygen gas for one minute in a reactive ion etching system (FA-1, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing), and preserved in the ultrapure water, before the cell culture.

The contact angles were measured between PDMS and medium by the contact angle analyzer (Phoenix-300, Meiwafosis Co., Ltd., Tokyo, Japan), before and after the oxygen plasma ashing.

Cell Culture

The culture dish, which consists of 6 cylindrical wells of 35 mm diameter, was used for cell culture (Fig. 4). The PDMS disk, which has micro ridges on the upper surface, was placed

in the bottom of each well, and preserved in medium of D-MEM (Dulbecco's Modified Eagle Medium). C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the experiment. Cells were seeded on the culture plate with the medium of D-MEM at density of 1.5×10^3 cells per cm^2. Fetal bovine serum (FBS) was added to the medium with the volume rate of 10 percent. Cells were cultured in the incubator for one week.

The deformation of cells near the micro ridges was observed with an inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo) every three hours in 24 hours.

The angle between the longitudinal axis of the cell adjacent to the ridge and the longitudinal direction of the ridge was measured (Fig. 5).

3. RESULTS

Figs. 6 & 7 exemplify the results of the measurement of the micro grooves of the manufactured molds. The data of the depth of the grooves fluctuate in the range of 30 percent of the target dimension.

Fig. 4: Cell culture on disk of PDMS with micro ridges dipped in the dish of six wells.

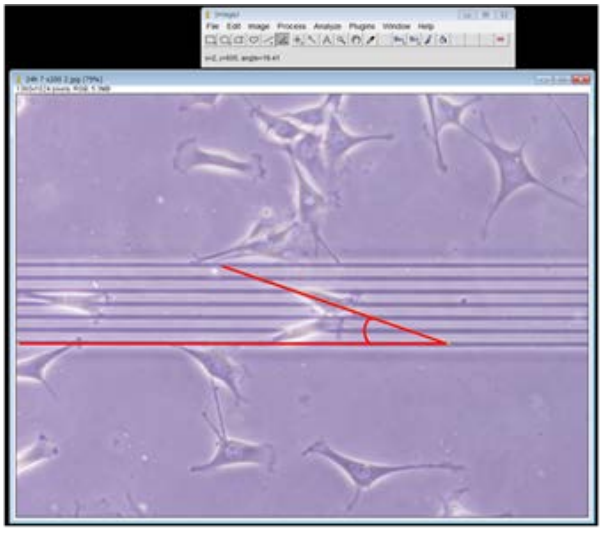

Fig. 5: Angle between longitudinal axis of cell and longitudinal direction of ridge. Dimension from left to right is 0.5 mm.

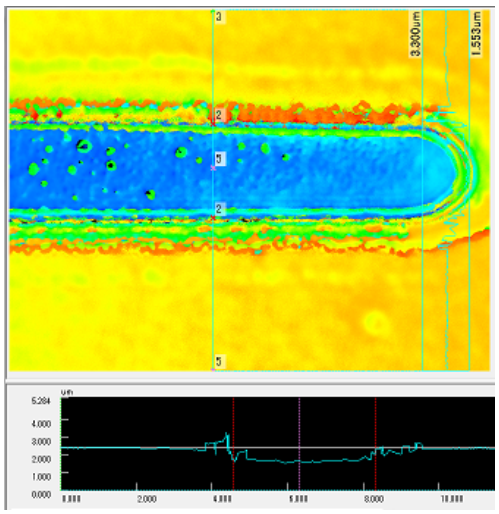

Fig. 6: Dimension of groove of mold measured with laser microscope. Lower figure of tracing along vertical direction in upper figure (dimension from left to right is 0.015 mm) shows depth of 0.0007 mm.

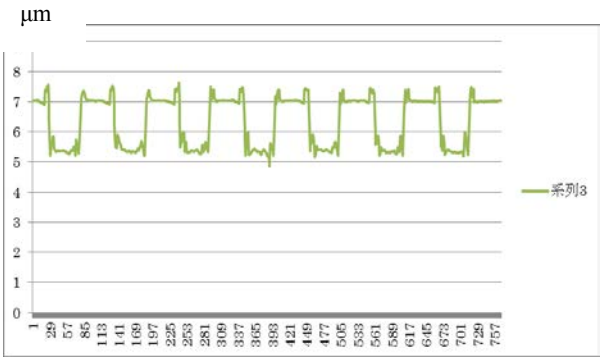

Fig. 7: Cyclic gap in tracing of surface of mold with laser microscope shows depth of grooves of 0.0015 mm.

Fig. 8(A): Drop of medium on PDMS before oxygen plasma ashing.

Fig. 8(B): Drop of medium on PDMS after oxygen plasma ashing.

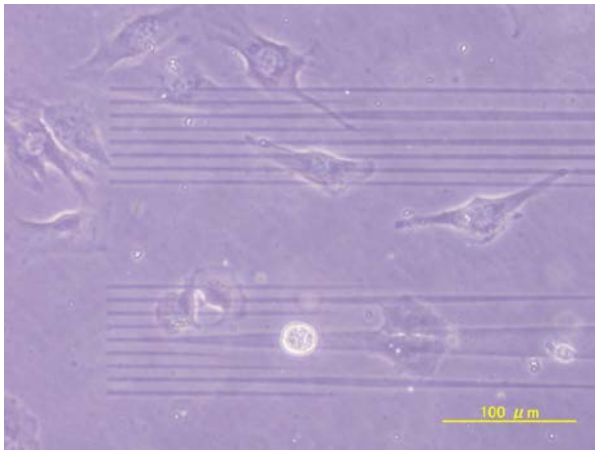

Fig. 9: 24 hours of cultivation on ridges of 0.0003 mm height. Cell spreads over the ridges of 0.0003 mm height.

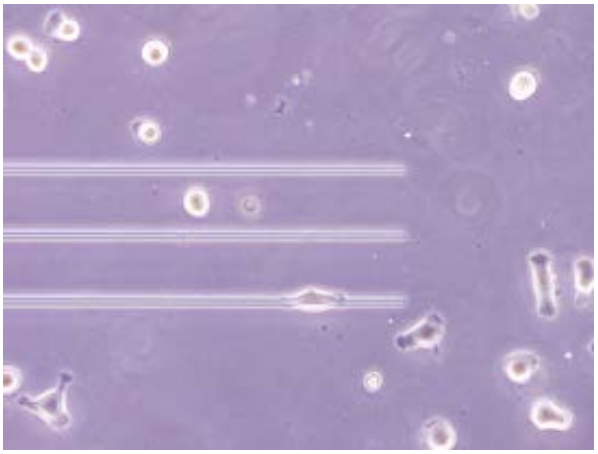

Fig. 10: 6 hours of cultivation near ridge of 0.0015 mm height. A cell adheres on the top of the single ridge, and extends along the longitudinal direction of the ridge.

Fig. 8 shows the contact between PDMS and the medium. The figure shows that the angle decreases (Fig. 8B) after oxygen plasma ashing on the surface of PDMS. The angle decreases when the surface of PDMS becomes hydrophilic.

Figs. 9-13 exemplify cells near the micro ridges during seven days of cultivation. Dimension from left to right is 0.5 mm in Figs. 9-13. The figure shows following behavior of cells. The cells adhere over the low ridge of 0.0003 mm height (Fig. 9). The cells stay on the top of the ridge lower than 0.0025 mm, and extend pseudopodium along the longitudinal direction of the ridge (Figs. 10 & 11). The cells, on the other hand, fall down into the valley of the interval of 0.007 mm between the ridges higher than 0.003 mm, and extend along the longitudinal direction of the valley (Fig. 12). Near the end of the ridge, some cells extend pseudopodium into the space between ridges (Fig. 13).

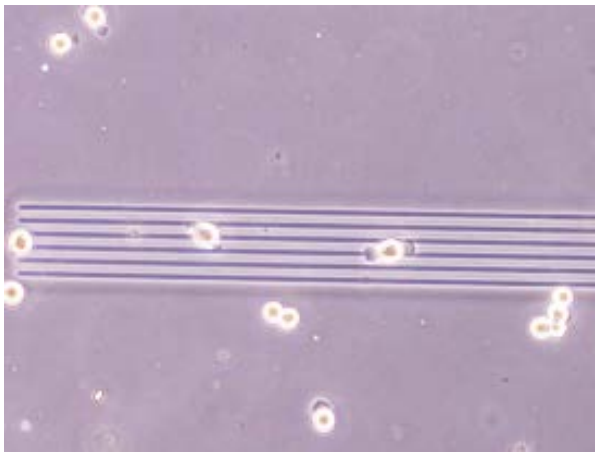

Fig. 11: 3 hours of cultivation on ridges of 0.0007 mm height. A cell adheres on the top of the ridge, and extends along the longitudinal direction of the ridge.

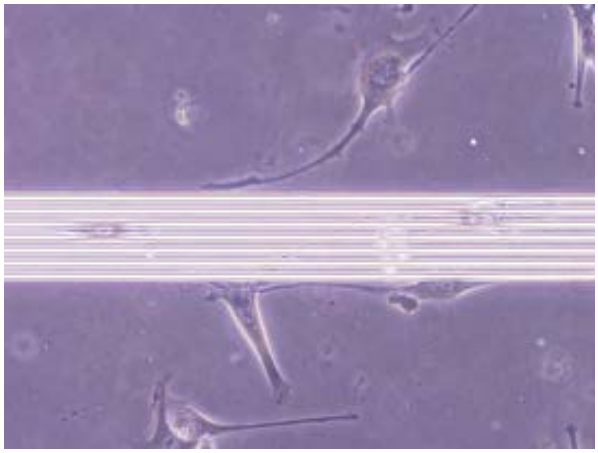

Fig. 12: 2 days of cultivation near ridges of 0.0035 mm height. Cells fall down into the valley between the ridges, and extend along the longitudinal direction of the valley.

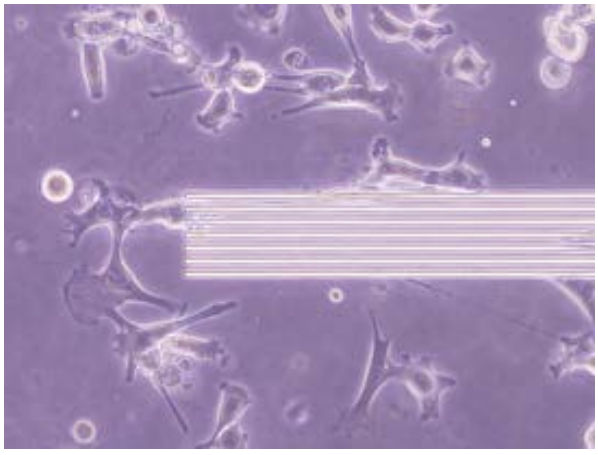

Fig. 13: 3 days of cultivation near ridges of 0.0035 mm height. Cell extends pseudopodium into the valley between ridges. Dimension from left to right is 0.5 mm.

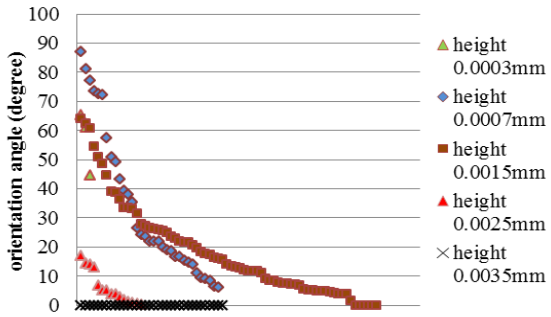

Fig. 14(A): Angle between longitudinal axis of cell and longitudinal direction of ridge at various heights of ridges.

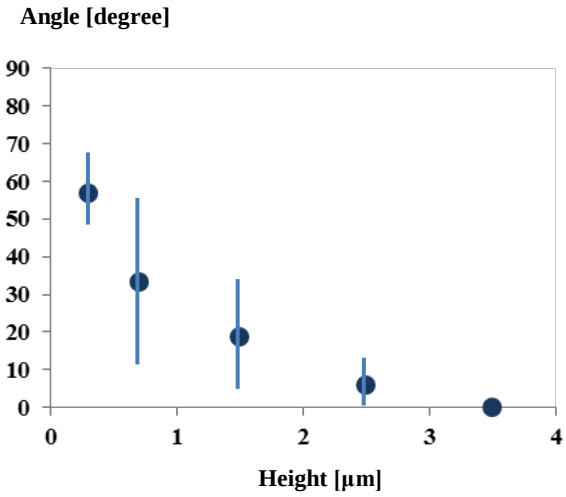

Fig. 14(B): Mean angle (point) between longitudinal axis of cell and longitudinal direction of ridge in relation to height of ridge. The vertical bar shows standard deviation.

The distribution of the angle between the longitudinal axis of cell and the longitudinal direction of ridge with the variation of height of ridges is shown in Fig. 14(A), in which data are plotted in the order of decreasing. Between the ridges with height of 0.0035 mm, cells are dropped in the groove between the ridges (Fig. 12), so that the angle is zero. Most of the angles distribute in the area lower than 45 degree, which shows the tendency of orientation of cell along the longitudinal direction of the ridge.

The data are integrated to the mean value (point) and the standard deviation (bar) in Fig. 14(B). When the data are distinguished from those at the height of 0.0035 mm, the most of data at the height of 0.0015 mm and 0.0025 mm are lower than 45 degree.

The experimental results show that cells adhere on the top of the ridge and align to the longitudinal direction of the micro ridges with the height between 0.0015 mm and 0.0025 mm.

4. DISCUSSION

In the previous studies, the micro pattern was designed to control the orientation of cell in the tissue *in vitro* [14-17]. The adhesion of cell was controlled with hydrophilic and hydrophobic micro-domains of polymer [18]. The tissue was designed to control interaction between cell and polymer *in vitro* [6]. Control methodology for orientation and proliferation of cells has a potential to be applied to a bio-actuator [19].

To make orientation of cells in the cultured tissue, several morphologies were applied to the cell culture in the previous studies: a micro capillary of glass and a groove of sub-millimeter. In these experiments, the cells orient just along the wall, and cells are forced to be aligned to the longitudinal direction of the space sandwiched between walls [13].

The biological cells, on the other hand, might sense micro morphology of the surface smaller than their own dimension through their cytoskeleton or membrane [10, 11]. The effect of the curvature of grooves of micro meter order on the behavior of cell was studied in the previous study [20]. Morphology of nanometer order of the surface might affect behavior of cells [21, 22].

In the most of previous studies, there were two ways to design the micro structure of scaffold for cell culture: nanometer - structure with molecular structure, and sub-millimeter-structure with surface machining. Between these two dimensions, micrometer-structure might be controlled with the photolithography technique. In the present study, the effect of micrometer order of morphology on the orientation of cells has been studied.

The experimental results show that cells move and elongate according to the ridge. Although the single ridge is not enough to make orientation of cells, multiple ridges affect the cells orientation [13]. The surface morphology of micrometer affects cells behavior. The differentiation of cells can be accelerated with micro ridges [13].

The experimental results with C2C12 show that cells adhere on the top of the ridge and align in the longitudinal direction of the micro ridges with the height between 0.0015 mm and 0.0025 mm (Fig. 15(A)). The ridge, which is lower than 0.0003 mm, is not enough to make orientation of the cell. The ridge, which is higher than 0.003 mm, is too high for the cell to stay on the top of the ridge.

Between the higher ridges, the cells fall down into the valley between ridges, and extend along the valley (Fig. 12). Near the higher single ridge, the cells tend to align along to the wall of the ridge (Fig. 15(B)).

The alignment of cells affects that of neighbor cells. A cell may rotate to make parallel alignment to the neighbor cell. A cell may also make parallel alignment through proliferation. Through the mechanism, the alignment of the single cell governs orientation of cells in the tissue.

To study on the effect of surface micro morphology of the solid scaffold on the alignment of the cell, the alignment of the longitudinal direction of the cell has been measured within 24

hours after adhesion of the cell. If cells proliferate to the confluent state, interaction between cells is dominant, and each environmental effect on orientation of cells cannot be distinguished each other.

In the differentiation of C2C12, cells fuse and make myotube. The alignment of cells may govern the alignment of myotube.

In the case of solid surface of the scaffold, the micro grooves might give a space for the flow of the medium between the cell and the scaffold. That might make better condition to grow the tissue.

5. CONCLUSION

The effect of micro ridges on orientation of cultured cells has been studied *in vitro*. Several lines of micro ridges have been fabricated on a transparent polydimethylsiloxane disk with photo lithography technique. 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.

6. ACKNOWLEDGMENT

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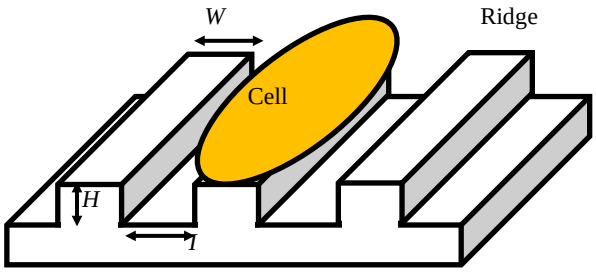

Fig. 15(A): Orientation of cell on low ridge.

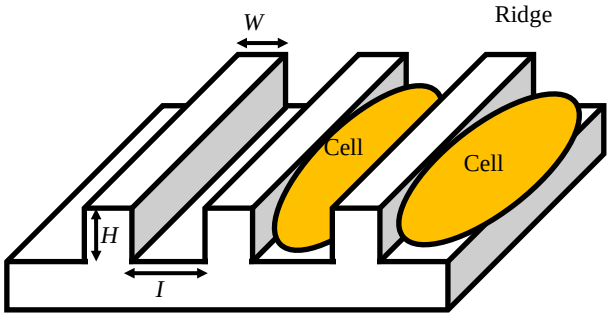

Fig. 15(B): Orientation of cell between high ridges (middle) and near ridge (right). *W*, width; *I*, interval; *H*, height.

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